Mitogenic Factors in Coronary Vascular Smooth Muscle Proliferation in Salmonids

by

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B.Sc. of Fu Dan University, Shanghai, China, 1975

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in the department of Biological Sciences

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Mitogenic factors in coronary vascular smooth muscle proliferation in salmonids

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Abstract

Ocean-going salmonids naturally develop coronary lesions consisting primarily of intimal smooth muscle cells even though they have high levels of a potentially beneficial compound, ω-3 polyunsaturated fatty acids (PUFA). My goal was to identify factors, including PUFAs and their metabolites, that affect coronary vascular smooth muscle proliferation in vitro and, therefore might be implicated in the process of coronary lesion development. I validated and used [³H]thymidine to measure mitotic activity of medial smooth muscle in coronary explants in vitro.

With this method, a three-fold increase in [³H]thymidine incorporation occurred after acute, gentle abrasion to the coronary artery of fish in vivo. In addition, in fish challenged to swim maximally over 3 months, [³H]thymidine incorporation in the coronary artery explants was 2.7 times greater than that in a group of slower swimming control fish. I suggest that these data are consistent with the hypothesis that coronary lesions in salmonids are primarily initiated as a result of mechanical injury linked to stressful activities in the fish's lives.

Concentration-response studies were performed by incubating pure PUFAs with rainbow trout coronary explants for 48 h. 20 μM arachidonic acid (AA) caused an approximately a 7-fold increase of [³H]thymidine incorporation compared with control conditions and with eicosapentaenoic acid (EPA) and eicosatrienoic acid (ETA) at the same concentration. The marked stimulation by 20 μM AA could be completely or partially inhibited by equimolar amounts of EPA and ETA, respectively. Four PUFA metabolites were also
studied. A thromboxane A₂ analogue, a prostaglandin I₂ analogue and prostaglandin F₂α all significantly stimulated [³H]thymidine incorporation, but quantitatively less than 20 µM AA. Leukotriene C₄ had no effect. I suggest that the availability of various PUFA families and their metabolites have a profound effect on coronary vascular smooth muscle proliferation of rainbow trout.

Fatty acid composition in phospholipid (PL) of fish gills was measured. AA compositions from phosphatidylinositol (PI) of gills were 1.5- and 3.7-fold higher than EPA in juvenile and mature coho salmon, respectively. These data suggested that gills of mature fish might prefer ω-6 PUFA to ω-3 PUFA as their major PUFA component in PI for eicosanoid production, even though there were high levels of ω-3 PUFA in fish tissues.

To conclude, vascular injury, possibly associated with environmental stress, may initiate coronary proliferation in salmonids, with ω-6 PUFA (AA) and its metabolites being powerful vascular smooth muscle mitogens. Even though EPA can limit the mitogenic effect of AA, the reason why salmonids accumulate lesions despite having high levels of ω-3 PUFA is suggested to be the preference of gill tissue for ω-6 over ω-3 PUFA as their PL component, setting the stage for a proportionately higher production of AA-derived metabolites. Further studies are needed to link these observations medial vascular smooth muscle proliferation to the production of coronary lesions involving intimal vascular smooth muscle proliferation.
Acknowledgements

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### Abbreviations

<table>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>bl/s</td>
<td>Body length/second</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ETA</td>
<td>Eicosatrienoic acid</td>
</tr>
<tr>
<td>G-6-PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LT</td>
<td>Leukotriene</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized low density lipoprotein</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipid</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>TES</td>
<td>N-tris [hydroxymethyl] methyl-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TX</td>
<td>Thromboxane</td>
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Part One

Mechanical injury and environmental stresses as stimulatory factors in vascular smooth muscle proliferation
I Introduction

The accumulation of atherosclerotic lesions in the coronary arteries of migratory salmon is a common and natural phenomenon (Robertson et al. 1961). The lesions are characterized by intimal proliferation of vascular smooth muscle with a disrupted elastic lamina (Moore et al. 1976a, 1976b). Furthermore, vascular smooth muscle cell proliferation has been recognized as a key event in the initiation and progression of coronary atherosclerosis in mammals (Ross and Glomset 1976; Ross 1986; Nilsson 1986; Morisaki et al. 1988a; Jackson et al. 1988) and current concepts of the etiology of coronary arterial lesions focus on the role of intimal vascular smooth muscle cell proliferation. My aim in Part One of this thesis is to identify stimulatory factors which stimulate vascular smooth muscle proliferation in fish. By addressing this general issue in a salmonid model of coronary lesions, I expected that my thesis would provide insights into the basic mechanisms underlying vascular smooth muscle proliferation which may then be applied to the issue of lesion formation in coronary arteries.

What follows is a general introductory perspective, a literature review, and a point of reference for the research performed in Part One.

I.1 Coronary circulation

Since the thesis focuses on the lesions confined to the coronary arteries of fish, a description of the coronary circulation provides my starting point.
A. Mammals

The human coronary artery arises from a root of the aorta (Gregg 1950). After passage through the capillary beds, the coronary blood returns to the right atrium. The coronary artery provides the entire supply of oxygen to the myocardium. The coronary artery blood pressure is essentially the same as the aorta. The mean aortic blood pressure is about 100 mmHg in the human coronary artery. Thus, the changes in aortic pressure generally evoke parallel changes in coronary blood pressure. Intra-arterial pressure is an important risk factor in coronary artery disease in mammals.

B. Fish

The coronary circulation of salmonids has a cranial (cephalad) origin of circulation. One to three pairs of branches from the efferent branchial arteries meet medially to form the hypobranchial artery, which exits from the gill arches. The coronary artery is a main branch from the hypobranchial artery, and is typically a single vessel running along the surface of the ventral aorta and bulbus arteriosus (Farrell and Jones 1992). This single artery bifurcates near the bulboventricular junction to form the right and left coronary arteries (Fig. 1) which are distributed throughout the compact myocardium of the ventricle. The outer compact myocardium of the ventricle, which represents about 40% of the entire ventricle in salmonids, relies on the coronary artery for its oxygen supply (Farrell et al. 1985). The inner spongy myocardium of the ventricle and the atrium are adequately supplied by oxygen contained in the venous blood (Farrell et al. 1985). Consequently, at
Fig. 1. The coronary artery on the *bulbus arteriosus* in fish. The main coronary artery which lies on the surface of the ventral aorta and bulbus arteriosus divides before reaching the ventricle. The superficial location of the coronary artery on a very elastic vessel is a central point to injury hypothesis proposed in the thesis. Coronary explant samples were taken from main coronary artery prior to bifurcation.
least part of the salmonid heart can still receive oxygen if the coronary flow is stopped.

In fish, blood ejected from the ventricle through the bulbus arteriosus first passes through the gills before reaching the coronary artery. Thus, the blood pressure in the coronary artery is about 60-75% of intraventricular pressure. The mean arterial blood pressure in the fish coronary artery is 30-40 mmHg (Farrell and Jones 1992).

I.2 Structure of the coronary arterial wall

Morphologically, the normal structure of the coronary arterial wall is similar in all vertebrates including salmonids and humans (Massey 1984); it is essentially a thick, muscular, elastic tube consisting of three tissue layers: the intima, media, and adventitia (Fig. 2).

The intima includes an internal elastic lamina bounded by an endothelium on its internal side and by the internal elastic lamina on its outer surface. The endothelium consists of a single layer of endothelial cells whereas the internal elastic lamina consists of a highly fenestrated sheet of elastic fibers that looks much like a fishman's net when laid out flat. This openness of the meshwork of elastic fibers permits relatively easy movement of substances and cells between the lumen, intima and media. Both the movement of substances, such as elastin, hormones, growth factors, and cells, such as vascular smooth muscle cells from the media to the intima, and macrophages from lumen to media, are important processes during the progression of atherosclerosis.
Fig. 2. The normal vertebrate artery wall. The coronary arterial wall is composed of three tissue layers which are the intima, media, and adventitia. The intima contains a single layer of endothelial cells and a highly fenestrated sheet of elastic fibers, and the openness of the meshwork of elastic fibers permits relatively easy access of substances and cells from the lumen and endothelium to the media, vascular smooth muscle. The media is at the middle of arterial wall, which is constituted of several layers of smooth muscle cells. The smooth muscle cells have two phenotypes. Under normal physiological conditions, the smooth muscle cells have a contractile phenotype and display contractile function and they may become synthetic phenotype after stimulation by mitogens (Ross 1993). The adventitia is the outer layer of coronary arterial wall. The adventitia consists principally of bundles of collagen fibers and elastins, some capillaries, arterioles and nerves together with some smooth muscle cells, fibroblasts, macrophages and lymphocytes.
Endothelium

Internal Elastic Lamina

Intima

Media

Adventitia
The media is bounded by the internal elastic lamina on one side and by the external elastic lamina on the other. The media consists of numerous layers of smooth muscle cells in mammals. There is less vascular smooth muscle in salmonids, presumably because of the lower intra-arterial pressure in the coronary artery.

The outer layer of coronary arterial wall, the adventitia, is bounded by the external elastic lamina and the outer surface of the artery. The adventitia in mammals consists of large amounts of connective tissue (principally bundles of collagen fibers) and elastins, some capillaries, arterioles, and nerves together with some smooth muscle cells, fibroblasts, macrophages and lymphocytes. In fish the external layer is mostly collagen.

A feature distinguishing human coronary arteries from fish arteries is the presence of a thickened intima (Hamby 1979; Stary 1989). The endothelium in the human coronary artery does not rest directly on the internal elastic lamina, but overlies a deeper intimal zone consisting of small numbers of smooth muscle cells and their extracellular matrix products. The intimal smooth muscle cells toward the luminal side tend to lose their contractile features; those below retain a contractile form and tend to adopt a predominantly longitudinal orientation (McConnell et al. 1982). Similar structures are found in a few other types of artery that are not prone to atherosclerosis (e.g., the renal artery and the helicoid arteries of the penis, McConnell et al. 1982; Banya 1990). Their appearance is regarded as a normal developmental event rather than a pathological feature. Nevertheless, intimal thickening of the coronary artery is thought to
Predispose certain sites to further change that may ultimately lead to overt atherosclerosis (Stary 1989).

I.3 Coronary artery disease in mammals

A. Description of coronary artery disease

Before dealing with coronary artery disease, I want to explain briefly the terms of arteriosclerosis and atherosclerosis. Arteriosclerosis literally means "hardening of the arteries". It refers to a broad group of disorders that have in common thickening because of fibrous plaque formation in the lesion site and loss of elasticity of arterial walls. Atherosclerosis is a morphologic variant of arteriosclerosis and is characterized by the formation of fibrous plaques. The fibrous plaque consists of a raised focal plaque within the intima, having a core of lipid, smooth muscle cells and foam cells, and a cap of fibrous material. If the above atherosclerotic process happens in the coronary artery, it is termed coronary artery disease. This fibrous plaque can increase in size with time and may impede the flow of blood, particularly if the margins of the fibrous cap rupture or fissure, resulting in haemorrhage into the plaque, thrombosis and ultimately occlusion of the artery (Davies and Thomas 1984). This may cause sudden death from myocardial infarction. Coronary artery disease is the leading cause of death in North Americans.

The characteristics of coronary arterial lesions in mammals (fibrous plaque) are shown in Fig. 3c and are from the description provided by Ross (1976). A core of lipid and necrotic debris is typically presented at the base of the
fibrous plaque, a cellular region containing smooth muscle cells and macrophages lies above, and a denser fibrous layer of connective tissue containing occasional attenuated smooth muscle cells forms a cap. The smooth muscle cells and macrophages of the cellular region frequently show intercellular accumulation of cholesterol and cholesterol esters. A characteristic cell morphology observed in these regions is the foam cell, formed predominantly from macrophages (Watanabe et al. 1989), but also sometimes from smooth muscle cells that have taken up large quantities of lipid (Wolfbauer et al. 1986). Death and disintegration of foam cells can lead to accumulation of extracellular lipid and necrotic debris in the core. Crystallization of cholesterol and calcification also may occur.

In lesions the fibrous cap may completely cover the endothelium, though some degree of endothelial loss is usually seen (Davies et al. 1988). Thrombi may form over areas of denuded endothelium or where plaques develop fissures. These thrombi may, with further growth of the plaque, subsequently become incorporated as a constituent. The fibrous plaques, by projecting into the arterial lumen, may impede the flow of blood. Recent data have shown that most of the sudden deaths from myocardial infarcts are due to ruptures or fissures, particularly in the margins of the fibrous cap where there are more macrophages, resulting in haemorrhage into the plaque, thrombosis and occlusion of the artery (Davies and Thomas 1984).

B. Etiology of coronary lesions

Several hypotheses have been proposed to explain the initiation of coronary lesions in mammals. However, no single mechanism has yet emerged to
adequately explain all aspects of the initiation process. There are, nevertheless, a sequence of recognizable events associated with initiation and progression of the plaque. These events are described in this section and some of the major proposed mechanisms are described in the following section.

**a. Morphological features of coronary lesion development**

The development of coronary artery lesions in mammals is characterized by three recognizable processes: 1) In the earliest recognizable lesion, the so-called fatty streak, an aggregation of lipid-rich macrophages and T-lymphocytes occurs within the innermost layer of the intima and the internal elastic lamina is split and fragmented; 2) In intermediate lesions, macrophages form and become foam cells and smooth muscle cells migrate from the media into the intimal layer where they proliferate; and (3) Influx of cells into the intima continues and proliferation leads to the advanced lesion, the fibrous plaque, with a core of lipid and necrotic debris overlaid by smooth muscle cells and foam cells and capped by connective tissue. The earliest recognizable alteration to the vessel wall (Fig. 3a) is a change in endothelial permeability and adhesion of platelets. According to the response to injury theory, several different sources of injury to the endothelium (mechanical, toxins, viruses, etc.) can lead to endothelial cell dysfunction, resulting in a series of morphological and biochemical alterations (Ross 1993). Monocytes and T lymphocytes then attach to the endothelium and migrate between the endothelial cells into the subendothelial space under the influence of the growth regulatory molecules and chemo-attractants released by altered endothelium, its adherent leukocytes, platelets, and possibly by underlying
smooth muscle cells. The internal elastic lamina, which is under the influence of growth-regulatory molecules, fragments and splits.

As the process continues, smooth muscle cells migrate to the intima through the fenestrae or breaks in the internal elastic lamina. These smooth muscle cells, no longer in their contractile state, proliferate in the intima. Smooth muscle cells form the predominant cellular component of the intimal lesion. Meanwhile, migrating cells reach further beneath the arterial surface, where the monocytes become macrophages. The macrophages which accumulate lipids, e.g. oxidized low density lipoprotein (oxLDL), are called foam cells. Macrophages and foam cells together with the accompanying lymphocytes form the fatty streaks (Faggiotto and Ross 1984; Ross 1990, 1993) (Fig. 3b). These fatty streaks often form at sites of pre-existing collections of intimal smooth muscle (Thomas et al. 1983b). Thereafter, continued cellular influx and proliferation in the intima leads to the more advanced lesion, which is distinguished by its fibrous character (Schwartz et al. 1986; Ross 1993). The plaque is composed of proliferated smooth muscle cells and foam cells, with a lipid core containing calcium deposits. As the plaque expands, the lumen is narrowed and the media at the base of the lesion is thinned (Fig. 3c).

b. Factors involved in the progression of coronary lesions

During the progression of coronary artery lesions, growth factors, cytokines and other chemicals promote cell recruitment, migration and cell proliferation and control lipid and protein synthesis (including extracellular matrix proteins). For example, basic fibroblast growth factor (bFGF), thromboxane A2 (TXA2), endothelin-1 (ET-1), interleukin-1 (IL-1), tumor
Fig. 3 Coronary lesions in fatty streak (a), intermediate lesions (b), and fibrous plaque (c) in a mammalian model (Ross 1976). After injury endothelial cells become dysfunctional. The changes of the arterial wall following the endothelial cell dysfunction cause a series of morphological and biochemical alterations. In the earliest recognizable lesions (Fig. 3a) there is an alteration of endothelial permeability and adhesion of some blood cells, e.g. platelets, to the site of injury. Monocytes and T lymphocytes attach to the endothelium and migrate between the endothelial cells into the subendothelial space. The migration of these cells are influenced by a variety of the growth-regulatory molecules and chemoattractants released by the altered endothelium, the adherent leukocytes, platelets, and possibly by the underlying smooth muscle cells. The internal elastic lamina, which is also under the influence of growth-regulatory molecules, fragments and splits. As the process continues (Fig. 3b), smooth muscle cells migrate to intima through the fenestrae or breaks in the internal elastic lamina and proliferate in intima. In addition, migrating white blood cells reach further beneath the arterial surface, where the monocytes become macrophages. The macrophages which accumulate lipids (oxLDL) are called foam cells. Macrophages, and foam cells together with the accompanying lymphocytes, form the fatty streaks. These fatty streaks often form at sites of pre-existing collections of intimal smooth muscle. Continued cellular influx and proliferation in the intima leads to the more advanced lesion (Fig. 3c), which is distinguished by its fibrous character, and is called the fibrous plaque. There is a lipid core with calcium deposits at the center of the plaque. The middle layer of the plaque is composed of proliferated smooth muscle cells and foam cells. Elastin and collagen cap covers the plaque. As the plaque expands, the lumen is narrowed, the media at the base of the lesion is thinned.
Fig. 3a

Fig. 3b

Fig. 3c
necrosis factor α (TNFα), and transforming growth factor β (TGFβ) can all induce smooth muscle cell and monocyte proliferation in the intima (Owen 1986; Morisaki et al. 1988b; Hultgardh-Nilsson et al. 1991; Reidy et al. 1992a; Ross 1993). Besides a mitogenic action, TXA₂, and TGFβ are also chemo-attractants, and are thought to induce medial smooth muscle cell migration into the subendothelial space of the coronary artery wall, and promote monocyte and lymphocyte chemotaxis (Davies and Thomas 1984; Nilsson et al. 1986; Munro et al. 1988).

Smooth muscle cells become a principal cellular component of the coronary lesion (Ross 1993). To do this, they undergo a profound functional alteration. Under the influence of the chemo-attractants and mitogens, as demonstrated in vivo or in vitro in cell culture (Campbell et al. 1989, 1990), smooth muscle cells can be induced to migrate, proliferate, and become highly active in synthetic phenotype, losing their normal contractile function (Thyberg et al. 1990; Ross 1993). In the synthetic phenotype, the contractile apparatus of the cell becomes rudimentary, the Golgi apparatus and rough endoplasmic reticulum become prominent, and a greatly augmented production of external lamina and extracellular matrix components occurs. The main extracellular matrix components synthesized by smooth muscle cells are collagen, elastin and proteoglycans (Severs and Robenek 1992). Various classes of extracellular matrix components are not only responsible for the overall structural integrity of the vessel wall, but can also have profound influences on cell adhesion, motility, and differentiation (Burgeson 1988; Kjllen and Lindahl 1991). Recently, a particularly important discovery was that synthetic smooth muscle cells synthesize bFGF, a potent smooth muscle cell mitogen in mammals that is released by injured smooth muscle cells (Sato et
al. 1991; Edelman et al. 1992; Reidy et al. 1992a, 1992b, 1993). Historically, platelet-derived growth factor was thought to be the key mitogen necessary for proliferation of smooth muscle cells following injury (Ross et al. 1976, 1986; Hart et al. 1988; Raines et al. 1990; Heldin and Westermark 1990). However, there is also data from several laboratories suggesting that it might not be important for smooth muscle cell replication in vivo (Majesky et al. 1985, 1987). This factor is a potent mitogen for many cells in vitro. Thus, synthetic smooth muscle cells may create a self-stimulating ("autocrine") cycle of proliferation and production of extracellular matrix components. This process may also become augmented by insulin-like growth factor, endothelin (Yanagisawa 1988), prostaglandins (PG) (Owen 1986), leukotrienes (LT) (Nilsson et al. 1985), IL-1, and TNFα (Ross 1993). Consequently, the synthetic activity of the smooth muscle cells determines the matrix content of the lesion to a large degree.

C. Possible mechanisms

a. Cholesterol and lipid infiltration hypothesis

The lipid infiltration hypothesis shares a long history, dating back to discussion of the role of the stimulation of plasma constituents into the intima (Steinberg et al. 1989). In its modern form, this hypothesis envisages the key event in atherosclerosis as infiltration of lipids (lipoproteins) from the serum through the endothelial barrier, with deposition into the subendothelial connective tissue. The discovery that low density lipoprotein (LDL) can undergo oxidative modification in the artery wall and that macrophages possess a scavenger receptor through which they avidly take up
oxidatively modified LDL (oxLDL) has been pivotal to the development of the cholesterol hypothesis in its current form (Steinberg et al. 1989). This oxidation modification results in dramatic alterations to the properties of LDL. The oxLDL acts as a chemo-attractant for monocytes, inhibits macrophage migration, and is cytotoxic (Steinberg et al. 1989). From this standpoint atherosclerosis can be envisaged as starting with elevated levels of LDL in the serum which, through an unsaturable endothelial transport process, leads to delivery of large amounts of LDL to the intima. Then monocytes/macrophages rapidly take up oxLDL via scavenger receptors and become foam cells. The endothelium may become damaged owing to the cytotoxic effect of oxLDL. It is now well-established that the modification of lipoproteins increases atherogenic potential. The common pathogenic element of the modification is their rapid uptake by macrophages in the vascular wall. Implicit in the lipid hypothesis is the assumption that advanced lesions evolve only from fatty streaks; the response to injury hypothesis (see below), by contrast, does not preclude other origins. In the lipid hypothesis, endothelial injury is envisaged as important in both the progression of the fatty streak to the fibrous plaque, and the initiation of the lesion.

b. The monoclonal hypothesis

This hypothesis is based on the notion that the smooth muscle cells within atherosclerotic lesions are often monoclonal in origin. Thus smooth muscle cell proliferation in atherosclerosis could represent a neoplastic process, possibly involving a mutational event. The etiological factors responsible could include virus (Hajjar 1991; Benditt et al. 1983) or carcinogens (Majesky
1985). The monoclonal hypothesis originated with a study of glucose-6-phosphate dehydrogenase (G-6-PD) isozyme types in vascular smooth muscle (Benditt and Benditt 1973). Smooth muscle cell samples of atherosclerotic plaques from a group of Afro-American females heterozygous for G-6-PD were found frequently to be monotypic, i.e., they displayed just one of the two G-6-PD isoforms. Smooth muscle cells from the surrounding vessel wall expressed both isozymes. There is, however, a distinction to be drawn between monotypism and monoclonality. Whereas a monoclonal origin is one possible explanation for monotypism, it is not the only one; G-6-PD type could be an indirect marker for other characteristics of selective survival in the proliferative response (Fialkow 1974).

c. The lymphocyte and immune hypothesis

Apart from the monocytes/macrophages that play multiple and complex roles in atherosclerosis, lymphocytes are found in atherosclerotic lesions, and these cells potentially provide another major source of cytokines and mediators for events important in lesion development. One hypothesis (Lopes-Virella et al. 1990) suggests that monocytes could be activated by circulating immune complexes; the resulting release of monokines would increase adhesivity of the endothelial plasma membrane and monocyte migration, macrophage transformation, foam cell formation, and endothelium damage. Once macrophages are established within a lesion, whether by this or other mechanisms, release of interleukin-1 from these cells could lead to adherence and activation of lymphocytes. Meanwhile, the activated lymphocytes produce interferon a, which stimulates interleukin-2 release (Pober et al. 1982, Hansson et al. 1989). Interleukin-2 has the capacity to augment
adhesion to the endothelium and trigger release of growth factors from macrophages. These growth factors and mediators are potent inducers of smooth muscle cell migration and proliferation. The final outcome of a primary immune reaction may be smooth muscle cell proliferation, matrix production and the other events of lesion growth. In the more common forms of atherosclerosis, the immune mechanism may become involved as lesions develop, but overall, probably plays a lesser role.

d. The response to injury hypothesis

An explanation of atherosclerosis that is widely accepted as a possible mechanism is based on the "response to injury hypothesis" attributed originally to Virchow (1856) and developed and elaborated over the last two decades by Ross and his colleagues (Ross and Glomset 1976; Ross 1986, 1988, 1989, 1993). This hypothesis in its modern form proposes that the initiating event in atherosclerosis is injury to the endothelium (Ross 1986). Endothelium injury may take a variety of forms, and the resultant effect is the release of growth factors into the intima and subintimal zone. The growth factors act as chemo-attractants and mitogens on smooth muscle cells, stimulating them to migrate from the media into the intima, proliferate, and adopt synthetic properties. The source of the growth factors may be platelets, macrophages, endothelial cells, smooth muscle cells, or any combination of these cell types.

The concept of endothelial injury is deliberately broad to encompass a spectrum of changes ranging from the stripping of the entire lining of a vessel, at one extreme, to retraction of still-adherent adjacent cells, through
to more subtle, morphologically imperceptible, functional alterations, at the other extreme. The well-established risk factors, high blood pressure, cigarette smoking, and cholesterol, are all plausible causes of such damage via mechanical stress, toxic effect, and altered membrane properties, respectively. Endotoxin (Harker et al. 1976), immune complexes (Fust et al. 1978), viruses (Hajjar 1991), and other factors reported to promote atherogenesis are all envisaged, according to the response to injury hypothesis, as primary mediators of endothelial dysfunction (Ross 1993).

Experimental studies on animals, using transmural balloons and nylon catheters, have shown that very extensive erosion of the endothelium, or repetitive desquamation in the same area, will induce smooth muscle cell proliferation. Meanwhile, endothelial dysfunction causes the secretion of growth factors by endothelial cells, and increased expression of adhesive molecules on their luminal surfaces. Intimal smooth muscle cells, whether newly migrated from the media or resident in the normal thickened intima or human coronary arteries, respond to the growth factors released by endothelial cells, platelets, and/or macrophages by modulating their synthesis. As a result, large quantities of extracellular matrix components are manufactured, resulting in the accumulation of fibrous material and bulk growth of the plaque. Further growth of the established plaque may occur by the same paracrine processes or may, in part, become a self-perpetuating process enhanced by autocrine mechanisms.
I.4 Coronary lesions in salmonids

A. Description of lesions

Coronary arterial lesions were first observed in Pacific salmonids by Robertson et al. (1961). Since that time these lesions have been characterized morphologically and quantitatively in a variety of salmonid species under different conditions (Van Citters and Watson 1968; Maneche et al. 1972; Moore et al. 1976a, b; House et al. 1979; Schmidt and House 1979; Farrell and Munt 1981; Eaton et al. 1984; McKenzie et al. 1978, 1985; Farrell et al. 1986, 1990, 1992).

Coronary arterial lesions in salmonids typically involve myointimal hyperplasia similar to that found in the early stages of mammalian arteriosclerosis when vascular smooth muscle predominates (House and Benditt 1981). As the lesions progress there is an extensive intimal proliferation of vascular smooth muscle which can form a 50% lumenal occlusion of the main coronary artery (Maneche et al. 1972; Moore et al. 1976a; McKenzie et al. 1978; House and Benditt 1981) (Fig. 4).

The main intimal component of the salmonid lesions is the vascular smooth muscle. Smooth muscle cells likely enter the intima through the fenestrae or breaks in the internal elastic lamina, since the internal elastic lamina in the fish lesions is invariably split, fragmented or absent (Moore et al. 1976a; House and Benditt 1981). Collagen and elastin are also present in the intima and form subendothelial fibroelastic caps that cover the proliferative smooth muscle at the lesion site. Significant fatty deposits and calcification
Fig. 4. Coronary lesions in fish. Smooth muscle cells proliferate and enter the intima through the fenestrae or breaks in the internal elastic lamina. The internal elastic lamina in the lesions is invariably split, fragmented or absent. Collagen and elastin are present at intima and form subendothelial fibroelastic caps that cover the proliferative smooth muscle cells in the lesion site. Smooth muscle cells, collagen, and elastin are the main constituents in the plaque at the lesion site in fish. In salmonid lesions, significant lipids deposits and calcification are absent. The initial stage of the lesion is small and focal, but later lesions may merge and increase in size, sometimes occluding the lumen of the main coronary artery by as much as 50%.
are, however, absent. The early forms of lesions may be small, but later they may merge and increase in size, finally occluding the lumen of the main coronary artery to a significant degree (Moore et al. 1976a, 1976b). Thus, the similarity between coronary arterial lesions in salmonids and mammals lies on the proliferation of intimal vascular smooth muscle; the differences are the absence of significant fat and calcium accumulations that characterize the plaques of mammalian atheroma (Massey 1984; Farrell and Jones 1992). The extent of knowledge of lesion morphology in fish, especially at different stages of development, is far less than that which is available for mammals.

It is now clear that coronary lesions are prevalent among salmonid populations and the severity of the lesions is remarkably high in mature, migratory fish (Robertson et al. 1961; Van Citters and Watson 1968; Maneche et al. 1972; Moore et al. 1976a, 1976b; House and Benditt 1981; Farrell et al. 1986; Saunders and Farrell 1988; Farrell 1990). Fewer than 5% of individuals within a population of mature, migratory salmonids are coronary lesion-free. In addition to the majority of individual fish possessing lesions, typically, 66% to 80% of the length of the main coronary artery has some form of intimal smooth muscle proliferation. Furthermore, this smooth muscle proliferation is sufficient to narrow portions of the lumen of the artery. A severe level of coronary lesions normally takes 2-5 years to develop in wild Atlantic salmon, and similar time (29 months) in faster growing, cultured Atlantic salmon (Saunders and Farrell 1988).

Coronary lesions are less severe in non-migratory salmonids, and absent or less severe in other fish species (Santer 1985). Farrell et al. (1992) made observations on five species of elasmobranchs having different lifestyles (fast
swimming sharks Lamna nasus and Isurus oxyrinchus; less active, but continuous swimming elasmobranchs Squalus acanthias and Prionace glauca; and a benthic species, Raja nasuta). No lesions were found in the main coronary artery of these shark species. However, lesions were found in smaller intravascular arteries and almost every branching point of the main coronary arteries (Garcia-Garrido et al. 1993). The reason why lesions are found in the main coronary artery of salmonids but not in elasmobranchs may be related to the different properties of the bulbus arteriosus in the former and the conus arteriosus in the latter (Farrell and Jones 1992).

B. Etiology of lesions

The reason for coronary arterial lesion formation and development in salmonids is far less clear than for mammals. Although several hypotheses have been proposed for the cause of coronary lesions in salmonids, their experimental support is often either fragmentary, superficial or preliminary. The following reviews these hypotheses.

a. Sexual maturation

Robertson et al. (1961) first suspected that sexual maturation was the primary factor in lesion development. This hypothesis was founded on the observation that lesions, which are absent in juveniles, appeared in maturing and mature fish. House et al. (1979) found that lesions increased in juvenile trout following injections of human chorionic gonadotrophin, estradiol, or testosterone. Lesions were also found in sexually precocious steelhead trout (Schmidt and House 1979). However, because well-developed lesions were
found well in advance of maturation in Atlantic salmon (Farrell et al. 1986; Saunders et al. 1992), it has been concluded that sexual maturation is probably only a secondary factor in lesion etiology (Farrell et al. 1986).

b. Diet and plasma cholesterol

Since lesions can be found in immature fish, the roles of diet and blood cholesterol have been considered in studies of salmonid arteriosclerosis. Cholesterol and, specifically, the LDL fraction are central factors in some forms of coronary disease in mammals (Brown and Goldstein 1984). Indeed, the cholesterol and lipid infiltration hypothesis is still regarded by some researchers as the central mechanism in the etiology of mammalian lesions (see above). Moore et al. (1976a), with no experimental evidence, first suggested that early forms of coronary lesions in salmonids may be related to diet alone. Salmon have high blood cholesterol levels, up to five times greater than those normally found in mammals (Larsson and Fange 1977; Farrell and Munt 1983). A preliminary study (Farrell and Munt 1981) showed that a dietary cholesterol supplement was associated with an increase in the number of lesions in brook trout (Salvelinus fontinalis). In addition, lesion proliferation was found to parallel the natural oscillations in low ratio of plasma high density lipoprotein:low density lipoprotein (HDL:LDL) of maturing chinook salmon (Oncorhynchus tshawytscha) in the Great Lakes (Eaton et al. 1984). A role of dietary cholesterol in lesion formation was found in experiments where the dietary cholesterol intake was experimentally altered in Atlantic salmon (Farrell et al. 1986), but overall its effect was rather small. Furthermore, since fish lesions lack the deposits
typical in mammalian lesions, it seems unlikely that the cholesterol/lipid infiltration mechanism is important in fish lesion etiology.

Epidemiological studies in humans have indicated that ω-6 PUFA and its metabolites play an important role in atherosclerosis formation in humans (Moncada and Vane 1979; Morisaki et al. 1983; Herold and Kinsella 1986; Pomerantz and Hajjar 1989; Lands 1992), and ω-3 PUFA and its metabolites can prevent this disease (Dyerberg et al. 1978; Dyerberg and Bang 1979; Kromhaut 1985). Nothing is known about the role of ω-3 and ω-6 PUFA and their metabolites on atherosclerosis in salmonids. However, migratory salmon have very high levels of ω-3 PUFA at the same time as severity of lesions is increasing. Given the findings for mammals, this seems enigmatic.

c. Growth rate

A study of the progression of coronary lesions in all lifestages of wild and cultured Atlantic salmon found a striking correlation between the growth of the fish and the progression of coronary lesions (Saunders et al. 1992). The immediate conclusion was that lesion progression was simply time-dependent. However, a closer analysis of the data suggested that the major factor promoting lesions in Atlantic salmon was probably a factor directly related to their growth rate. For example, when Atlantic salmon enter the marine environment to begin an exponential growth phase, coronary lesions increase correspondingly. Furthermore, when Atlantic salmon are grown under culture conditions, they grow and mature faster and attain a similar level of lesion prevalence as wild salmon but in a shorter time period
(Saunders et al. 1992). The mechanism underlying the correlation between lesion development and fish growth is unknown.

Mature Atlantic and Pacific salmon and steelhead trout all have high levels of coronary lesions when they reach their spawning ground (Robertson et al. 1961, Van Citters and Watson 1968, Manache et al. 1972; Farrell et al. 1990). The majority of spawning fish have some level of coronary artery lesions and, in most individuals, the lesions are classed as severe since they significantly occlude the lumen of vessel. Unlike Pacific salmon, Atlantic salmon and steelhead trout do not necessarily die after spawning; some Atlantic salmon may spawn a second time, and exceptional individuals may spawn as many as six times (Ducharme 1969). Van Citters and Watson (1968) reported that when steelhead trout (Oncorhynchus mykiss) returned to the ocean after spawning, the coronary lesions had regressed naturally and almost completely. This raises the question of what happens to the high level of coronary lesions after spawning: do they regress or increase in number and severity? Some authors concluded that the lesions of steelhead trout (Salmo gairdneri) (Van Citters and Watson 1968; Schmidt and House 1979) and Atlantic salmon (Maneche et al. 1972) undergo regression after spawning. However, in a detailed study of coronary arterial lesions in all life-history stages (including immature, mature, and postspawned Atlantic salmon), Saunders and Farrell (1988) found no evidence of lesion regression. Saunders et al. (1992) found that postspawned Atlantic salmon had a high incidence of severe lesions similar to those in sexually mature fish. Farrell et al. (1992) investigated lesions in both wild repeat-spawning steelhead trout that were caught at high sea, and in wild and cultured steelhead trout that had been held in sea pens for up to 1 year after maturation, and found a high
prevalence and severity of coronary lesions in postspawned fish. Combined, these findings challenge the concept of lesion regression in salmonids.

d. Mechanical injury

In an attempt to explain how lesion development is related to growth rate, and acknowledging the importance of the vascular injury hypothesis in the etiology of mammalian lesions, Saunders and Farrell (1992) proposed that coronary lesions in salmonids were primarily initiated as a result of general mechanical injury to the coronary artery wall. This suggestion forms the basis for some of the experimental work in this thesis. The proposed mechanism is described in the following text and the accompanying Fig. 5.

Central to the hypothesis is that mechanical injury to the coronary artery wall results from stressful activities in fish life, e.g. avoiding predation and feeding, and cumulative injury may lead to coronary lesion formation. More successful fish (i.e., fast growing) are those with greater success at feeding and predator avoidance. An inherent behaviour common to feeding and predation avoidance is burst swimming, probably to near their maximum capacity. Such extensive physical exertion is quite common place in the fish world, although it is not a daily event for most humans.

The proposed linkage between swimming to near exhaustion and injury to the coronary artery wall is entirely speculative at this time but is founded on the following facts. Exercise in fish causes increases in cardiac output, arterial blood pressure and blood catecholamine levels. However, it is important to distinguish between the two major forms of exercise in fish:
Fig. 5. Possible mechanism of lesion formation. Environmentally stressful activities in fish life, e.g., avoiding predation and seeding may induce hypertension. The hypertension could lead to overdistension of the main arterial outflow tract from the heart. Because the coronary artery lies on the surface of the highly compliant bulbus arteriosus and ventral aorta, the hypertension may in turn physically distort and disturb the coronary artery, altering its blood flow pattern and perhaps inducing vascular injury. The accumulative injury in coronary artery may cause lesion formation.
Increased feeding and predation in marine phase

Increased stresses (natural)

Increased growth rate

Hypertension

Injury to coronary artery

Over distention of bulbus arteriosus

PROMOTION
sustained or prolonged aerobic swimming and burst swimming. The cardiovascular changes associated with prolonged aerobic swimming were first comprehensively documented for rainbow trout by Kiceniuk and Jones (1977). In their study cardiac output increased threefold, from 17.6 ml/kg/min to 52.6 ml/kg/min at the maximum aerobic swimming speed. The increase in cardiac output occurred primarily through a doubling of cardiac stroke volume. The increased cardiac output was greater than the associated reduction in vascular resistance and mean blood pressure in the ventral aorta and the dorsal aorta both increased by about 50% and 20%, respectively (Kiceniuk and Jones 1977; Randall and Daxboeck 1982; Axelsson et al. 1989; Axelsson and Fritsche 1991).

Furthermore, the increase in arterial blood pressure is greatest near exhaustion. Possibly correlating with the fact that catecholamines are released into the blood of aerobically exercising fish only near exhaustion. Thus, the pressor response observed in aerobically exercising fish as they approach exhaustion is in part due to an α-adrenergically mediated systemic vasoconstriction which is well documented for fish (Primmett et al. 1986; Milligan et al. 1989).

Burst swimming typically exhausts fish in several minutes. The cardiovascular changes are quite different to those associated with sustained, aerobic swimming. There is an initial bradycardia and decrease in cardiac output (Stevens et al. 1974; Farrell 1981) which is thought to protect the gills' blood vessels from rupture as the skeletal muscle forcefully contracts, limiting systemic blood flow (Farrell and Jones 1992). With time the tail beats become less forceful, the bradycardia gives way to tachycardia, cardiac
output increases, catecholamine levels increase 10-100 fold and arterial blood pressure increases (Primmett et al. 1986; Milligan et al. 1989). Axelsson and Nilsson (1986), in their study of cod, showed that stressful swimming, e.g. burst swimming, caused catecholamine levels to rise greatly. In trout, repeated burst swimming, the levels of noradrenaline increased from 0.74 nM at rest to 85 nM, and adrenaline increased from 0.91 nM at rest to 212 nM (Primmett et al. 1986). Although the increase in cardiac output during burst swimming and recovery is less marked than with aerobic swimming, the increase in arterial blood pressure is impressive, presumably reflecting a significant systemic vasoconstriction due to catecholamine release.

During frenzied feeding activities and predator avoidance, fish rely heavily on burst swimming. Therefore, these behaviors are expected to be associated with significant and repeated elevations in blood pressure. However, no one has yet made measurements of blood pressure during such behavioural activities.

The linkage between the acute hypertensive event and damage to the coronary arterial wall is equally speculative at this time but rests on three important facts. Foremost, the outflow from the ventricle enters a very elastic chamber, the bulbus arteriosus. Second, there is a significant increase in stroke volume of the heart (more than heart rate) with exercise in fish. Third, there is a significant increase in ventral aortic blood pressure (more than dorsal aortic blood pressure).

The linkage between acute hypertension and coronary arterial damage is probably not related to changes in the coronary inter-arterial blood pressure.
Because blood pressure does not increase markedly in the dorsal aorta and dorsal aortic blood pressure is representative of the intra-arterial pressure in the coronary artery, it seems unlikely that changes in dorsal aortic pressure cause significant injury to the coronary artery wall. Instead, injury more likely relates to external stretching and disruption. The coronary artery lies on the outside wall of the elastic *bulbus arteriosus*. The increase in cardiac stroke volume and ventral aortic blood pressure will both lead to excessive distention of the *bulbus arteriosus* with each cardiac cycle. It is likely that these large changes in *bulbus* volume distort and possibly injure the coronary artery. The nature of the injury to the vessel wall is unclear at this time. It may involve a disruption to the coronary endothelial layer through either a mechanical disturbance or altered shear rates, if the blood flow pattern in the coronary artery is changed. What is known is that the endothelium of the trout coronary artery was easily disturbed and removed when coronary vascular rings were being threaded with very fine wires for isometric tension measurements (Small et al. 1990). Following damage to the endothelial, it would seem likely that a similar sequence of events as occurs in mammals would occur in salmonids involving release of chemo-ttractants and mitogens.

*bulbus arteriosus* is an elastic chamber composed primarily of smooth muscle and elastic tissue (Licht and Harris 1973). The wall of the *bulbus* is very elastic being 32 times more distensible than the human thoracic aorta (Satchell 1991). The elasticity is in part due to some 10 radial septa that run the length of the chamber, each bearing radial arranged bands of elastic fibers and smooth muscle. The inner ends of these insert on to thicker longitudinal bands which form the inner ends of the septa. Priede (1976) suggests that these serve to counteract the effect of LaPlace’s law, i.e. that the tension of the wall is inversely proportional to the radius of the chamber. As the *bulbus* expands, the radial and longitudinal elastic fibres are stretched. In contrast with *bulbus arteriosus*, the coronary artery is a muscular artery and probably is less elastic then *bulbus arteriosus*. So when the *bulbus arteriosus* expands radially with each heart beat, the main coronary artery (by lying on the surface of the *bulbus arteriosus*) must by necessity stretch longitudinally. It is envisaged that the excessive distension of the *bulbus arteriosus* that likely accompanies swimming causes excessive longitudinal stretch of the coronary artery and injury to the vessel wall (Saunders et al. 1992).
from the damaged endothelium and resulting in smooth muscle cell migration and proliferation. The specific nature of these chemicals in fish is completely unknown. In this thesis, I perform novel studies on potential mitogens for vascular smooth muscle proliferation in fish.

There is no direct experimental support for the above hypothesis regarding the proposed linkages between environmental stresses, swimming, elevated ventral aortic blood pressures, *bulbus arteriosus* overdistension and injury to the coronary artery. However, there is an empirical observation which is consistent with these ideas. The observation is the different pattern of coronary lesion accumulation in elasmobranches compared with salmonids. Elasmobranches spend all their lives in the marine environment rather than just the majority of the lives as in migratory salmonids. However, they do not accumulate lesions in the main coronary artery (Farrell et al. 1992). Instead they accumulate small, less severe lesions at branch points in the main coronary artery and in intra-ventricular arteries at sites of high mechanical stress. Thus, lesions in sharks are associated with regions of the coronary artery that are subject to either distension or altered blood flow patterns. The absence of coronary lesions in the main sections of the coronary artery may relate to the differences of the wall structure between *bulbus arteriosus* and *conus arteriosus*. The *conus arteriosus* is a muscular chamber between the ventricle and the *ventral aorta* in elasmonbranches. The coronary artery lies on its surface and has a similar anatomic position as the salmonids. A layer of cardiac muscle encircles and overlies the conal wall. The *conus* also has several sets of valves. Thus, when the ventricle discharges blood into the *conus*, the series of valves and the weak contraction of conal cardiac muscle result in little volume change in the *conus* (Farrell et
al. 1992). Certainly the conus is far less distensible than the bulbus. This being the case, episodes of acute hypertension, whether they are related to environmental stress or not, will not overdistend the elasmobranch conus, the main coronary artery is therefore left relatively undisturbed in terms of external mechanical disrupts and certainly lacks lesions.

C. Approaches for studying coronary vascular smooth muscle proliferation

The proliferation of vascular smooth muscle cells is the central feature of lesions in salmonids. Since there is virtually no experimental support for any major aspect of the etiology of coronary lesions in salmonids and a considerable amount of speculation, my intention was to focus specifically on this phenomenon and on the factors that might initiate vascular smooth muscle proliferation. To date there is no information available on this subject which is specific to fish.

In order to understand the effects of factors (physical or chemical) on coronary artery vascular smooth muscle proliferation, a method which can rapidly detect vascular smooth muscle proliferation is needed. Several methods are available, each with its own advantages, disadvantages and assumptions.

Histology is a commonly used method for quantifying lesions in arteries. It has an advantage in that the formed lesion is observed. However, light microscopy is limited to detecting the differences in lesion progression after a reasonably protracted time course. At best, explanations of observations can only imply association rather than causes and effects. In addition, I was
interested in factors initiating vascular smooth muscle proliferation. Electron micrographs can show the early stages of lesion development in which smooth muscle cells are in the proliferative stage, as indicated by the appearance of rough endoplasmic reticulum (Thomas et al. 1968). However, electron micrography is not an easy means of quantifying lesion progression. I wanted to quantify the effects on vascular smooth muscle proliferation in response to various experimental perturbations.

$[3^H]$thymidine incorporation during the S-phase of cell division is a well-established method for quantifying smooth muscle mitosis both in vivo and in vitro with mammalian models (Florentin et al. 1969; Thomas et al. 1968; Bevan et al. 1976; Goldberg et al. 1980; Thyberg et al. 1983; Jackson et al. 1988). The principle of the technique is based on isotope tracing in DNA synthesis. In proliferative smooth muscle cells, the DNA in the nucleus is replicated during the S-phase period of the cell cycle. Once DNA replicates during the S-phase, the cell cycle will automatically enter M-phase to undergo mitosis and subsequent cell division. $[3^H]$thymidine, a radioactive precursor of a thymine nucleotide that every cell uses exclusively for the synthesis of DNA, is employed as a marker of mitotic activity at the S-phase of the smooth muscle cell cycle. The more the $[3^H]$thymidine is incorporated into cells, the more active the mitotic activity is. Thus, $[3^H]$thymidine incorporation can be used as a quantitative marker of vascular smooth muscle proliferation. Vascular smooth muscle proliferation is of course a prerequisite for coronary lesion formation in salmonids.

The assumptions of this approach are that: (a) DNA synthesis occurs during vascular smooth muscle proliferation, and (b) vascular smooth muscle
proliferation contributes to lesion formation. These assumptions have been confirmed with mammalian models (Owen et al. 1986; Akopov et al. Morisaki et al. 1988b). Florentin et al. (1969) and Thomas et al. (1968) were able to detect increases in [3H]thymidine incorporation well before overt signs of smooth muscle cell proliferation were present in hyperlipidemic swine. This method was also used extensively to detect mitotic activity in a variety of explants, e.g., assessing the influence of the intensity of balloon catheter injury to rat thoracic aorta (Capron and Bruneval 1989), the DNA synthesis in isolated rat renal arteries exposed to growth factors (Boonen et al. 1991), the smooth muscle proliferation after injury to the rat carotid artery by balloon catheter (Clowes et al. 1989), the DNA synthesis from de-endothelialized rabbit aorta (Morisaki et al. 1989), and the effect of factors affecting proliferation of aortic explants (Owen 1986).

Since [3H]thymidine incorporation is a well established and accepted technique in the study of mammalian vascular smooth muscle proliferation and vascular smooth muscle is the predominant role cell type in fish lesions, this method was deemed appropriate to study vascular smooth muscle proliferation in fish coronary arteries. However, since the technique had never been used for fish vascular smooth muscle, I had to develop and validate the technique in this thesis.

The majority of studies of mammalian smooth muscle cell growth use subcultures of smooth muscle cells. These subcultured cells are usually in the synthetic rather than in the normal contractile state in the artery. In contrast, enzyme-dissociated cell cultures and primary explant cultures start with smooth muscle cells primarily in the contractile state (Campbell et al.
Enzyme dissociation presents a number of problems for routine use, not the least of which is the variable yield of viable cells associated, in part, with the variability of the enzymes used in digestion. Furthermore, the enzyme-dissociated cells no longer have the intercellular communication of their normal environment and the protein composition of the plasma membrane may be modified, perhaps causing abnormal behavior.

Primary explant cultures, on the other hand, provide a system in which the majority of cells are in their normal, special relationship with one another and with the surrounding matrix. In addition, the endothelium is still on the explant, therefore, any contribution it may have to vascular smooth muscle growth is incorporated. In view of these considerations, I selected the explant technique as the method of choice to provide a reliable system for studying the complex events associated with the initiation of vascular smooth muscle cell proliferation in fish coronary arteries. There is a disadvantage with the explant technique regarding extrapolation to lesion development. The explant technique only provides information about the early events (i.e., hours to days) in vascular smooth muscle proliferation and especially the switch from the contractile to synthetic states. Therefore, by using the explant technique, I similarly restrict the contribution I am making to the understanding of vascular smooth muscle proliferation as it applies to lesion formation in the coronary artery of salmonids.

At the outset of the study there were no reliable methods for isolating and culturing vascular smooth muscle from fish vessels. Furthermore, neither in vivo nor in vitro radiolabelling of smooth muscle growth has been developed for fish. Therefore, I developed and validated (radioautography) an in vitro
[\textsuperscript{3}H]thymidine labelling technique specifically for measuring vascular smooth muscle mitotic activity in coronary artery explants from salmonids. The details of this new method are described in Chapter II.

I.5 Objectives

The main aim of the present study is to investigate the initial factors which stimulate the vascular smooth muscle proliferation in the coronary artery of salmonids. This study is the first to investigate coronary artery vascular smooth muscle proliferation in fish in general, and at the cellular level specifically. To achieve this aim, the following research objectives were planned for Part One of the thesis:

(1) To develop an \textit{in vitro} assay of coronary smooth muscle cell proliferation in fish using [\textsuperscript{3}H]thymidine as a marker of DNA replication in tissue explants. This assay required the additional development of a micro-analysis technique for DNA determination.

(2) To perform \textit{in vivo} experiments in which there was either (a) direct mechanical damage to the coronary artery, or (b) environmental stresses likely to raise blood pressure, and then measure the response of the vascular smooth muscle explant.
II. Materials and Methods

II.1 General

A. Animal supply and husbandry

Three species of the salmonid genus *Oncorhynchus* were used in the experiments. All species of salmonids develop lesions in their coronary arteries, even though the severity of coronary lesions may differ somewhat at maturity among species. The majority of experiments used rainbow trout (*Oncorhynchus mykiss*), because they are reared commercially, are easy to obtain in the numbers required, and adapt well to captivity. The breeding stock at any hatchery tends to be limited, with the aquaculturist selecting for particular qualities in the fish, so these animals are genetically much more homogeneous than wild populations. This can be very useful in reducing the natural variability between animals. Rainbow trout (600-700 g), obtained from West Creek trout farm, Abbotsford, British Columbia, were used for (a) an estimation of the effects of the mechanical injury in experiment II.3.A and (b) burst swimming in II.3.C in Part One of the thesis, and (c) in Part Two of the thesis the effect of PUFAs in experiment II.A and (d) AA-derived eicosanoids in II.B on $[^{3}H]$thymidine incorporation into vascular arterial explants. Chinook salmon (*Oncorhynchus tshawytscha*) (55-95 g) raised by the Department of Fisheries and Oceans Canada, West Vancouver, were used to observe the effect of exercise training regimes on $[^{3}H]$thymidine incorporation into coronary arterial explants (see section II.3.B in Part One). Chinook salmon were used because I was able to piggy back on an ongoing
set of experiments being conducted at the Department of Fisheries and Oceans Canada, West Vancouver. The quality of these training experiments could not have been reproduced at SFU. Coho salmon (Oncorhynchus kisutch) parr (30-40 g) and adult (900-1,000 g), obtained from Capilano Hatchery, North Vancouver, British Columbia, were used for the determination of PUFA composition from gill phospholipid in fish from fresh and salt water (see section II.B in Part Two). Coho salmon from Capilano Hatchery were used because of the proximity of the hatchery to SFU.

Rainbow trout were held at Simon Fraser University under natural photoperiod in 2000 l fiberglass tanks supplied with dechlorinated municipal water. The water temperature varied seasonally. The water temperature was about 17-19°C in July and August for method development; about 11-13°C in November for Exp. II.3.A, B and C in Part One; and about 6-8°C in January and February for II.3.A and B in Part Two. During the period of holding, fish were fed daily to satiation with a commercial pellet diet (Oregon Moist).

B. In vitro assessment of vascular smooth muscle mitosis

A standardized method of in vitro [3H]thymidine incorporation during the S-phase of cell division was developed to estimate vascular smooth muscle mitosis and as an indicator of early lesion development in coronary arteries of salmonids.
a. Coronary arterial explant

Fish were killed by a sharp blow on the head before use. A long straight portion of the coronary artery together with a small piece of bulbus arteriosus and ventral aorta (on which the coronary artery lies) was removed and placed into sterile saline solution (0.7% NaCl). The bulbus arteriosus, ventral aorta and the external connective tissue layer (parenchyma of coronary artery explants) were removed before incubation. The bulk of the remaining tissue was medial vascular smooth muscle and intimal tissue. Any residual blood on the explant was washed off at this time. The vessel segment was then maintained in ice-chilled, modified Hanks balanced salts-1 (MHBS-1) (see "Basic medium" as below) before incubation.

b. Basic media

The basic medium was Hanks' balanced salts (HBS) (Sigma Chemical Co.). HBS buffered with 0.336 g/l of NaHCO$_3$ and 4.588 g/l of N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and supplemented with 0.065 g/l of the antibiotic gentamycin became modified Hanks' balanced salts-1 (MHBS-1). The MHBS-1 was gassed with 0.5% CO$_2$-99.5% air for 1 h at 15°C before its pH was adjusted to pH 7.6±0.1 (see optimization procedure below). The MHBS-1 and the above incubation conditions were used for the determination of the effects of mechanical injury and environmental stress on vascular smooth muscle proliferation.

A modified version of MHBS-1, MHBS-2, was used in Part Two of the thesis for determination of in vitro concentration-response effects of mitogenic
factors (e.g. PUFA) which were added to the medium. Since the metabolic processes that alter PUFA and that might affect cell division may take longer than the 13 h incubation period used in Part One, the incubation period was extended. R. Townley, under my supervision, performed a separate series of experiments to develop MHBS-2 for the longer (48 h) incubation period (R. Townley, BSc Honors Thesis, SFU, 1992). He has kindly allowed me to include these data in Appendix I. The medium (MHBS-2) was MHBS-1 plus 10% fetal bovine serum (FBS).

c. **Tissue explant culture conditions**

An aliquot of 0.35 ml medium was added into all the wells of a 96-well culture plate (Corning Glass Works) which was placed in a covered small box with inlet and outlet tubes on it. The inlet tube was connected to 0.5%CO2-99.5% air tank and the outlet tube was for exhaustion. The medium at the culture plate in the box was pre-incubated for 20 min at 37°C in an incubator (Johns Scientific 2010) with atmosphere of 0.5% CO2-99.5% air. At the end of this pre-incubation period, the vessel segment and 4 μl of [3H]thymidine in Part One and 8 μl in Part Two (1.0 mCi/ml, 70-85 Ci/mM, Amersham) were added to some of the 96 wells. The well culture-plate, containing the vascular tissue, was then incubated at 37°C in an atmosphere of 0.5% CO2-99.5% air. The pH in the culture medium was confirmed using a 24-well culture plate which contained the same medium as the 96-well plate in all the experiments. The 24-well medium was placed next to the 96-well plate in the same box of the incubator. The pH in the medium of the 24-well culture plate was measured only by pH meter not dye. The gas flow rate of 0.1 l/min for a 0.5% CO2-99.5% air mixture was found to maintain stably a pH of 7.6±
0.1 throughout the incubation period (13 or 48 h). The medium pH was checked every two hours and the pH was found to be consistent in all the 30 incubations used for method development. For the mechanical abrasion and swimming experiments, the medium pH was checked every 4 h within 13 h incubation period. For the stimulatory factor experiments, the medium pH was checked every 4 h for the first 12 h, once for the second 12 h, every 4 h for the third 12 h, and once for the fourth 12 h within 48 h incubation. Incubation was lasted 13 h in Part One of the thesis and 48 h in Part Two.

**d. Tissue treatment and DNA measurement**

The coronary tissue was homogenized in 80 μl of 2% perchloric acid (PCA) using a hand-held glass homogenizer (Radnoti Glass Technology, Inc. Monrovia, CA, USA) to disperse vascular smooth muscle cells. Following homogenization, a total of 1.4 ml of 2% perchloric acid was added and the homogenate was centrifuged at 10,000 rpm in an Eppendorf centrifuge at 4°C for 30 min. The supernatant fraction, which contained the acid-soluble [3H]thymidine that had not been incorporated into DNA, was discarded. This process was repeated six times to wash unincorporated [3H]thymidine from the tissue. The last washing solution was counted with the liquid scintillation counter to confirm that there was no radioactivity in the washing solution. The DNA from the dispersed vascular smooth muscle cells was extracted following the technique of Maniatis *et al.* (1982). The cell pellets were resuspended in 2 volumes of ice-cold tris-EDTA solution (100 mM tris and 10 mM EDTA, pH 8.0). Then 10 volumes of cell lysis solution (0.5 M EDTA (pH 8.0) and 100 μg/ml proteinase K) were added into the tris-EDTA solution. The suspension of lysed cells was placed in a 50°C water
bath for 3 h. The DNA was gently extracted three times with an equal volume of phenol. After centrifugation, the phenol generally forms the upper phase in these extractions because of the high salt concentration in the sample. The phenol layer was removed and as much of the interface as possible. Five volumes of 100% ethanol were added to precipitate the DNA. Following centrifugation (10,000 rpm for 30 min), the supernatant was discarded and the pellet was re-homogenized in 0.6 ml of DNA determination buffer (0.05 M NaH₂PO₄, 2.0 M NaCl, pH 7.4), and then mixed with 1 μg/ml of the fluorescent compound, bisbenzimidazole (Hoechst 33258) (American Hoechst Corporation) for DNA determination. The intensity of DNA bound fluorescence was determined using a Perkin-Elmer LS 50 fluorescence spectrophotometer linked to an IBM computer. The excitation wavelength was 345 nm (slit width 4 nm) and the emission wavelength was 475 nm (slit width 6 nm).

The procedure for micro-analysis of DNA was adapted from that described by Labarea et al. (1980). It is based on an enhancement of fluorescence seen when compound Hoechst 33258 binds to DNA. The DNA can be directly assayed. Also, the procedure can be used on crude homogenates in which the deoxyribonucleoprotein structure of chromatin has been dissociated with a high salt buffer, making the DNA fully accessible to the reagent. RNA does not interfere. 100-500 ng of DNA was the typical measurement range in my experiments.

For the DNA standard curve, a stock solution of 2 μg/μl was made from single stranded calf thymus DNA (Sigma Chemical Co.) (Fig. 6). A dilution series was performed resulting in DNA concentrations ranging from 0-1,000
Fig. 6. DNA standard curve.
ng/tube. Each tube had a total volume of 594 µl. To each tube 6 µl of Hoechst 33258 (100 µg/ml) was added resulting in a final concentration of 1 µg/ml. A DNA standard curve was run with every set of DNA quantifications. The fluorometer gave slightly different slopes and intercepts for the DNA standard curve every time when it was turned on. The coefficient of variation for the DNA standard curve in three determinations was 4.7%.

e. [³H]thymidine activity

After the DNA determination, all the solution was transferred into glass containing 20 ml of scintillation fluid (Beckman Ready Safe, Beckman Instruments, Inc. CA, USA). Radioactivity was typically counted for 5 min in a Beckman LSC scintillation counter (Beckman LS 3801). Counts typically ranged from 250-400 dpm for method development, from 500-800 dpm for mechanical injury and swimming experiments, and from 1,000-2,000 dpm for PUFAs and their metabolites added experiments. The background usually ranged from 90-120 dpm. The difference of counts between three experimental groups resulted from different culture media which were used for them and other factors such as chemical or physical stimulation factors, seasonal temperature variation of fish life, ages of fish and species of fish (see Discussion). The incorporation of [³H]thymidine was expressed as dpm/µg DNA.
II.2 Coronary explant of culture conditions

The incubation conditions can greatly affect \([^3\text{H}]\)thymidine incorporation into coronary arterial DNA. Thus, it was essential to define the optimum incubation conditions that I would use for the experimental perturbation. In this section the various incubation conditions were tested. The following approach was employed.

a. Type of culture media

A comparison was made between MHBS-1 and modified Eagles medium (both buffered with 20 mM of TES and 10 mM of NaHCO\(_3\), pH 7.6 and supplemented with 0.065g/l of gentamycin). Both media were gassed with 0.5% CO\(_2\)-99.5% air for 1 h at 15°C before their pH was adjusted with 0.1 N of NaOH.

b. Optimal pH

pH is an important determinant of \([^3\text{H}]\)thymidine incorporation. The optimum pH value using MHBS-1 was established by varying media pH values (pH 7.3, 7.5, 7.6, 7.7 and 7.9).

c. \([^3\text{H}]\)thymidine dose

The rate of \([^3\text{H}]\)thymidine incorporation into cultured coronary artery explant is related in part to the \([^3\text{H}]\)thymidine concentration in a medium.
Volumes of 1, 2, 4, and 8 μl of [3H]thymidine (1.0 mCi/ml; 70-85 Ci/mmole, Amersham Company) were tested in 0.35 ml of MHBS-1.

d. Incubation time

Fifteen incubation times, from 1 to 15 h, were used to determine the time required for maximal [3H]thymidine incorporation into coronary artery smooth muscle explant using MHBS-1. R. Townley also examined them at 24 h and 48 h using MHBS-2 (Appendix-2).

e. Incubation temperature and CO2 gas

The temperature (15°C) and CO2 concentration (0.5%) were selected for all experiments based on the known temperature for salmonid tissue culture (Fryer et al. 1965) and CO2 concentration in fish blood (Claiborne and Heisler 1984).

II.3 Validation of [3H]thymidine incorporation into vascular smooth muscle

Two types of tissue cultures were used in radioautography to locate the [3H]thymidine in arterial explants. One was 20 μM AA-treated coronary explants and the other was a non-AA-treated control explant. After incubation, half of the arterial segments explant were used for autoradiography. They were rinsed in cold saline and fixed in a saline formaldehyde solution (0.7% NaCl-4.5% formaldehyde) for 1 hour at room temperature. The fixed tissue was stored at 4°C for subsequent histology and radioautography. The remainder of the explants were used for
scintillation counting and DNA determination. In this way I was able to make a qualitative comparison between the usual appearance of radioactivity in histological slides and dpm/μg DNA.

Standard histological procedures were used for tissue embedding, section and slide mounting. After incubation, the small pieces of fish coronary explants were transferred to distilled water twice for 10 min to remove formaldehyde from the tissues. The tissues were then dehydrated through an ethanol series (30%, 50%, 70%, 95%, and 100% ethanol three times) and left in 100% ethanol for 10 min. Subsequently, 100% ethanol/Hemo-De (Fisher Scientific) (1:1, v/v) and Hemo-De were in turn used once and three times, respectively, to eliminate ethanol. Each treatment took 10 min. To replace Hemo-De with paraffin, the tissues were initially dipped in the mixed solution of Hemo-De/wax (1:1, v/v) once for 10 min; then they were transferred through three, 10 min changes of paraffin at 45°C. Serial arterial cross sections of 7 μm were cut from paraffin blocks and mounted on glass slides. Before radioautography, the slides were deparaffinized and hydrated with the reverse of the above dehydration process.

A dipping technique was used for radioautography. Several trials were attempted before the best results were obtained. Slides were dipped in Kodak NTB-2 liquified emulsion (maintained at 45°C in a water bath) and placed in a black box to expose the emulsion for 10 days at 4°C. The slides were developed in Kodak developer D19 at 10°C for 5 min. The slides were transferred to a stop bath Kodak SB5 at 10°C for 5 min for fixation, and washed in running water for 1 h. After staining with hematoxylin (Sigma Chemical Co.), the slides were dehydrated through an ethanol series (50%,...
70%, 95%, and 100% ethanol three times). After drying, the slides were mounted with Permount (Sigma Chemical Co.).

II.4 *In vivo* manipulation for *in vitro* of coronary explant culture to evaluate \[^3H\]thymidine incorporation into coronary arterial explants.

A. Effect of vascular injury

*a. Rationale*

Synthesis of DNA in arterial vascular smooth muscle is typically low under normal conditions but can increase in response to injury (Ross 1993). In this case, the increase could be because of either the injury itself, or a supply of exogenous growth factors. To evaluate the possibility that mechanical injury is an initiator of vascular smooth muscle proliferation, experiments were performed in which the outside wall of the coronary artery was gently rubbed with a blunt object *in vivo*. Arterial segments were then isolated after either 1, 2, or 3 days and incubated with \[^3H\]thymidine. Because the coronary artery is rather narrow in diameter in 1.2 kg fish (<1.0 mm) and difficult to access, it is impossible to perform the internal vascular damage techniques often used in mammalian work. The expectation was that gentle mechanical abrasion would damage the endothelium in particular, since previous experiments in this laboratory have demonstrated its fragile nature in fish coronary arteries (Small *et al.* 1990). Furthermore, since my working hypothesis was that the injury leading to coronary lesions may be derived from external rather than intravascular mechanical forces, the external gentle abrasion technique seems to be an appropriate approach.
b. Experimental design and protocol

Eighteen fish were subjected to gentle abrasion of the coronary artery under anaesthesia. Each fish was anesthetized with methanesulfonate (MS222) (1:5,000) and the main coronary artery which lies on the bulbus arteriosus and the ventral aorta was exposed (Farrell and Steffensen 1987). A small incision was made through the body wall and the pericardium in the region of the isthmus and the outside wall of the coronary artery was gently rubbed three times with the smooth outside edge of forceps. The abrasion surgery was performed by Dr. A.P. Farrell. The incision was closed with silk sutures and the fish recovered from the anaesthetic within minutes in an aquarium. The operation took 5-10 minutes and anaesthesia lasted no longer than 15 minutes. Groups of six fish were sacrificed after either 1, 2, or 3 days post-abrasion, and the abraded portion of the coronary artery was removed. A sham-operation was performed on an additional group of six fish. They were subjected to the same anaesthesia and surgical intervention, but the coronary artery was not rubbed. A further group of six control fish were not subjected to any surgical intervention before coronary artery removal.
B. Effect of sub-maximum and maximum aerobic swimming

a. Rationale

The swimming trials were performed to determine if different swimming regimes induce coronary vascular smooth muscle proliferation. Fish swimming at their maximum aerobic swimming speed would increase arterial blood pressure as a result of elevated cardiac output. According to my working hypothesis, the hypertension could lead to increased vascular smooth muscle mitotic activity if there was injury to the coronary vascular wall.

b. Experimental design and protocol

Chinook salmon were used as a part of a much larger and well-controlled exercise training experiment performed by Dr. Anders Kiessling and Dr. David Higgs at Department of Fisheries and Oceans, West Vancouver Laboratory. Fish were placed into identical 4-m$^3$ circular outdoor fiberglass tanks for exercise. Each tank was fitted with an inner fiberglass hoop creating a circular swimming channel 45 cm wide and 55 cm deep. Seawater was pumped into each tank. Supplemental water aeration was provided by Millipore tubing connected to compressed air. Water flow rates, temperatures, salinities and dissolved oxygen concentrations during the experiment ranged from 25-40 l/min, 8.2-12.3°C, 24-29% and 11-14 ppm, respectively.
Groups of fish were exposed to one of three training regimes: maximum aerobic swimming (2.8-2.9 body length (bl)/second (s)), sub-maximum aerobic swimming (1.5 bl/s) and low speed swimming (0.5 bl/s) were used. Fish in the maximum aerobic swimming group were challenged to swim to their maximum swimming velocity every second day. To do this fish were presented with stepwise increases in water velocity until they were exhausted. Typically the fish swam for 1-3 h before becoming exhausted. Exhausted fish lay on their side and could not swim. At all other times, the fish swam at 0.5 bl/s. Coronary explants were sampled from this group of fish after 3 months of the training regime. The sub-maximum swimming regime involved continuous swimming at 1.5 bl/s for 8 months. As control groups of continuously low speed swimming (0.5 bl/s) fish were trained for either 3 or 8 months. Eight fish were sampled from the end of each of the training regimes for evaluating \(^{3}\text{H}\)thymidine incorporation into coronary arterial explants.

Water velocities in different parts of the circular swimming channel were measured in each tank every second day using a flow meter (Braystoke BFM002, Valeport Marine Scientific, Devon, UK). No differences existed between the top and bottom of the water column.

C. Effect of burst swimming

a. Rationale

The purpose of the burst swimming experiment was to test if a short duration (3 days) but severe exercise stress (burst swimming) caused coronary
vascular smooth muscle proliferation. This treatment was chosen as one that was likely to induce a greater degree of elevated arterial blood pressure than that induced by the fast swimming regime. According to my working hypothesis, this higher level of hemodynamic stress would lead to a greater stimulation of vascular smooth muscle mitotic activity if the coronary vascular wall was injured.

b. Experimental design and protocol

Eight rainbow trout were chased by a stick in a circular tank until they were exhausted (<10 minutes to exhaustion). The exercise regime was implemented twice daily for 3 days. Coronary arterial explants were removed for *in vitro* [³H]thymidine incubation after the third day. Eight undisturbed, freely swimming fish were used as a control group.

D. Statistics

Statistical comparisons were performed using the Student’s *t*-test and a level of *p*<0.05 was taken as significant. Results are expressed as mean±standard error (SEM).
III.1 Optimum incubation conditions for $[^{3}\text{H}]$thymidine incorporation into vascular smooth muscle of a coronary explant from rainbow trout

The following results were used to establish the optimum incubation conditions for the media type, the buffer system, the $[^{3}\text{H}]$thymidine dose, and the incubation time for coronary explants from rainbow trout. I also confirmed the incorporation of the radiolabel into medial smooth muscle cell nuclei using autoradiography.

A. Radioautography

Two culture conditions were compared with radioautography: a control and a 20 mM AA-treated coronary explant (see Part Two of this thesis). The $[^{3}\text{H}]$thymidine incorporation was 255 dpm/μg DNA in the control, which was about ten times lower than that in AA-treated explants (2,688 dpm/μg DNA). This difference in $[^{3}\text{H}]$thymidine measured by liquid scintillation was consistent with the observations on the difference in the incorporation of the radiolabel in medial vascular smooth muscle nuclei as shown in the radioautographs.

A radioautograph of $[^{3}\text{H}]$thymidine incorporation into cell nuclei is showed in Fig. 7. Few black dots were found in vessel lumen of the control (Fig. 7a) and AA-treated tissue (Fig. 7b), which indicated that both control and AA-treated tissue had relatively clear backgrounds. Black dots in the background are
Fig. 7  Histological cross-sections of coronary artery explant incubated in MHBS-2 and 8 μCi of [3H]thymidine without AA as a control (Fig. 7a) and with AA (20 μM) as AA-treated explants (Fig. 7b) for 48 hr. The magnification was x180. The few black dots which represented [3H]thymidine molecules were found at lumen of coronary artery from both the control (Fig. 7a) and the 20 μM achidonic acid (AA)-treated explants (Fig. 7b). The black dots which gathered around smooth muscle cell nuclei of the AA-treated explants were much denser than that from the controls in the cytoplasm. Compared with the control the presence of black dots overlying smooth muscle cell nuclei in the media of the AA-treated coronary artery explant were much denser. Several big black spots which consisted of a large number of black dots formed by condensed isotope molecules overlie smooth muscle cell nuclei in the media. These radioautographs showed that [3H]thymidine was effectively incorporated into smooth muscle nuclei.
Fig. 7a

Media

Intima

Lumen

Nuclei

(shows no or a few [$^3$H] thymidine molecules in the nuclei)

55μM

Fig. 7b

Lumen

Media

Intima

Nuclei + [$^3$H] thymidine molecules

(shows high density of [$^3$H] thymidine molecules in the nuclei)

55μM
usually due to emulsion instability. There were some black dots in the cytoplasm, which represented either $[^3]$H]thymidine in the cytoplasm, or poor wash out despite the six washes with cold saline.

The black dots concentrated in the medial vascular smooth muscle cell nuclei from 20 \(\mu M\) arachidonic acid-treated explants were found more frequently than in the controls. In addition, several big black spots, which consisted of a large number of dots formed by condensed isotope molecules, overlaid nuclei in the AA-treated explants but not the control explants. The results of much denser $[^3]$H]thymidine molecules in medial smooth muscle cell nuclei from the AA-treated explants than from the control is consistent with the 10-fold higher isotope counts in the AA-treated explant. These results indicated that $[^3]$H]thymidine was effectively incorporated into smooth muscle cell nuclei. In other words, the DNA in vascular smooth muscle cells was actively replicating in the explants, especially after AA-treatment. These differences in DNA incorporation of $[^3]$H]thymidine were reflected in the liquid scintillation counting.

The majority of the label was incorporated into medial vascular smooth muscle as opposed to specific intimal tissues. Thus, events revealed by this explant technique reflect primarily a stimulation of vascular smooth muscle located in the media. No attempt was made to visualize whether any of the experimental perturbations caused vascular smooth muscle proliferation specific intimal to region of the artery.
B. Incubation time

Fig. 8 shows that with MHBS-1 coronary arterial smooth muscle began to incorporate $[^3\text{H}]$thymidine after a lag period of 3 h in primary culture. Afterwards, the $[^3\text{H}]$thymidine uptake into cellular DNA showed a typical exponential phase between 10-13 h incubation and a plateau phase thereafter. Because the explants showed no more growth after 13 h (the appearance of a plateau phase), the incubation was discontinued. The appearance of the plateau phase is an indicator of dedifferentiation in culture. R. Townley found a large incorporation after 48 h with MHBS-2 (See Appendix figure 1).

C. $[^3\text{H}]$thymidine dose

Volumes of 1, 2, 4, and 8 $\mu$Ci of $[^3\text{H}]$thymidine were tested. Fig. 9 shows the increase in effective incorporation (dpm/$\mu$g DNA) and isotope concentration ($\mu$Ci of isotope/0.35 ml of medium) between 1 $\mu$Ci to 4 $\mu$Ci. There was no further increase with 8 $\mu$Ci. R. Townley found that, with MHBS-2 and a longer incubation period of 48 h, 8 $\mu$Ci showed a significantly improved $[^3\text{H}]$thymidine incorporation over 4 $\mu$Ci (see Appendix 2).

D. Basic medium for $[^3\text{H}]$thymidine incorporation

There was a significant difference ($p<0.05$) in $[^3\text{H}]$thymidine incorporation between MHBS-1 (modified Hanks' balanced salts) and modified Eagles
Fig. 8. Time-dependent [\(^3\)H]thymidine incorporation into coronary artery smooth muscle of rainbow trout. Tissues were incubated in standardized MHBS-1 in a 15°C incubator with 0.5% CO\(_2\)-99.5% balance air for 15 h. Each point represents mean±SEM of results in 5 replicates (p<0.05).
Fig. 9. Dose-dependent $[^3]H$thymidine incorporation into coronary artery smooth muscle of rainbow trout. Tissues were incubated in 1, 2, 4 and 8 μCi of $[^3]H$thymidine (1.0 μCi/ml, 78 Ci/mM) with a standardized MHBS-1 in a 15°C incubator with 0.5% CO2-99.5% balance air for 13 h. Each point represents mean±SEM of results in 5 replicates. The * denotes a significant difference of $[^3]H$thymidine incorporation between 4 μCi and 1 μCi (p<0.05, evaluated by Student's t-test).
medium (MEM) (Fig. 10). MHBS-1 showed an approximately two-fold higher \[^{3}H\]thymidine incorporation of coronary artery explants.

E. pH and buffers

The \[^{3}H\]thymidine incorporation into vascular smooth muscle of salmon coronary arteries was sensitive to changes in pH of the MHBS-1 medium. Fig. 11 indicates that the optimum pH for \[^{3}H\]thymidine incorporation was pH 7.6 with rates of incorporation for pH 7.5 and 7.7 being not significantly different. However at pH 7.3 and pH 7.9 the dpm/μl DNA values were significantly lower (p<0.05) compared to that at pH 7.6.

In order to achieve a strong pH buffer capability for maintenance of pH 7.6± 0.1 in the medium, N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES) and NaHCO₃ were tested as components of the buffer system. \[^{3}H\]thymidine incorporation with 20 mM TES was significantly higher than that with 25 mM TES (p<0.05) (Fig. 12).

Similar to TES, the concentration of NaHCO₃ also had a significant effect (p<0.05) on \[^{3}H\]thymidine incorporation into vascular smooth muscle. Fig. 13 shows that \[^{3}H\]thymidine incorporation at 10 mM NaHCO₃ was significantly superior to 16 mM for smooth muscle growth in this medium. There was no significant difference in \[^{3}H\]thymidine incorporation between 12 mM and 10 mM NaHCO₃.
Fig. 10. Effect of MHBS-1 and MEM (MEM had Eagles medium basis with the same buffer system and pH value as MHBS-1) on [\(^3\)H]thymidine incorporation into coronary artery smooth muscle of rainbow trout. Tissues were incubated in the standardized MHBS-1 or MEM with 0.5% CO\(_2\)-99.5% air in a 15\(^\circ\)C incubator for 13 h. Each column represents mean±SEM of the results in 5 replicates. The asterisk indicates a significant difference of [\(^3\)H]thymidine incorporation from MEM (p<0.05, evaluated by Student's t-test).
Fig. 11. Effect of pH values on $[^3\text{H}]$thymidine incorporation into coronary artery smooth muscle of rainbow trout. Tissues were incubated in the standardized MHBS-1 with 5 different pH values, and with 0.5% CO$_2$-99.5% air in a 15°C incubator for 13 h. Each point denotes mean±SEM of the results in 5 replicates. Asterisks indicated significant differences of $[^3\text{H}]$thymidine incorporation from pH 7.6 ($p<0.05$, evaluated by Student's t-test).
Fig. 12. Effect of NaHCO₃ concentrations on [³H]thymidine incorporation into coronary artery smooth muscle of rainbow trout. Tissues were incubated in MHBS-1, varied NaHCO₃ concentrations, with 0.5% CO₂-99.5% air in a 15°C incubator for 13 h. Each column denotes mean±SEM of the results in 5 replicates. The asterisk indicates a significant difference of [³H]thymidine incorporation from 10 mM concentration (p<0.05, evaluated by Student's t-test).
Fig. 13. Effect of TES concentrations on $[^3$H]thymidine (1.0 μCi/ml, 78 Ci/mM) incorporation into coronary artery smooth muscle of rainbow trout. Tissues were incubated in standardized MHBS-1, varied TES concentrations, with 0.5% CO$_2$-99.5% air in a 15°C incubator for 13 h. Each column denotes mean±SEM of the results in 5 replicates. The asterisk indicates a significant difference of $[^3$H]thymidine incorporation from 20 μM concentration (p<0.05, evaluated by Student's t-test).
In view of the above results the following incubation conditions were routinely used with MHBS-1 in Part One of this thesis for the studies involving in vivo perturbation to fish:

Dose of $[^3\text{H}]$thymidine: $4 \mu\text{Ci}[^3\text{H}]$thymidine (1 mCi/ml, 70-85 Ci/m mole, Amersham) in 0.35 ml of medium.

Medium (MHBS-1): Hanks balance buffer salts with 20 mM TES, 10 mM NaHCO$_3$ and 0.065 g/l of gentamycin sulfate. pH 7.6±0.1.

Temperature: 15°C.

Atmosphere: 0.5% CO$_2$-99.5% air.

Incubation Time: 13 h

In Part Two of the thesis, MHBS-2 was routinely used for in vitro manipulations of the culture conditions. The dose of $[^3\text{H}]$thymidine in MHBS-2 was increased from 4 $\mu\text{Ci}$ to 8 $\mu\text{Ci}$ and the incubation time was increased from 13 h to 48 h.

III.2 In vivo manipulation for in vitro cultures of coronary explants for evaluating the effects of factors on $[^3\text{H}]$thymidine incorporation

A. Effect of vascular injury

As hypothesized, gentle mechanical rubbing of the outside of the coronary artery in vivo significantly stimulated $[^3\text{H}]$thymidine incorporation into
coronary artery explants. Fig. 14 shows a significant 3-fold increase (p<0.05) of [3H]thymidine incorporation two days after the mechanical injury. [3H]thymidine incorporation had declined by day 3 but was still significantly elevated compared with the sham-operated group. There was no statistical difference in [3H]thymidine incorporation between the treated and sham-operated groups on day 1. These results illustrate that the maximum proliferative response of the coronary smooth muscle likely peaked around 13 h after injury.

Interestingly, Fig. 14 also shows that there was no statistical difference of [3H]thymidine incorporation between the sham-operated and control groups. These results indicated that brief surgical intervention used here (e.g. netting, surgery and anesthesia), which are known to be stressful, had little effect on [3H]thymidine incorporation into coronary vascular smooth muscle.

B. Effect of maximum and sub-maximum aerobic swimming in vivo

As hypothesized in section I.4.B.d, the environmental stress of maximum aerobic swimming triggered vascular smooth muscle proliferation. Table 1 shows a significant increase (p<0.05) in [3H]thymidine incorporation into coronary explants from maximum aerobic swimming fish (2.8-2.9 bl/s) for 3 months compared with control swimming (0.5 bl/s). Sub-maximum aerobic swimming (1.5 bl/s) for 8 months had no significant effect (Table 1). The fish subjected to maximum aerobic swimming (2.8-2.9 bl/s) would have swam to exhaustion approximately 45 times over a 3 month period covering a total distance of about 442 km. In contrast, sub-maximum aerobic swimming fish
Fig. 14. Effect of mechanical injury on $[^3\text{H}]$thymidine incorporation into coronary arterial smooth muscle of rainbow trout. The injured coronary arterial explants were sampled at 1, 2 and 3 days after abrasion. The sham fish were treated under the same conditions as coronary artery injured fish with the exception of abrasion, and the vessel explants were also obtained at 1, 2, and 3 days after the surgery. The control fish were treated with neither abrasion nor surgery. The tissue from three groups were incubated in MHBS-1 for 48 h. $[^3\text{H}]$thymidine incorporation from mechanical abrasion $\Box$, sham operated $\bigcirc$ and control fish $\bigtriangleup$ is represented as the mean±SEM of 6 determinations. Asterisks indicate significant differences (p<0.05, evaluated by Student's t-test) from the sham operated fish.
DAYS AFTER SURGERY

INCORPORATION (dpm/µg DNA)

0 1 2 3 4

control

mechanical abrasion

sham operated

*
Table 1 Effect of Training Regimes on $[^3\text{H}]$thymidine Incorporation into Coronary Arterial Smooth Muscle of Chinook Salmon*

<table>
<thead>
<tr>
<th>Fish Group</th>
<th>Fish Swimming Speed</th>
<th>Duration</th>
<th>$[^3\text{H}]$thymidine (dpm/μgDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control(^1) (n=16)</td>
<td>0.5 bl/s</td>
<td>3-8 months</td>
<td>1435±242</td>
</tr>
<tr>
<td>Sub-maximum aerobic</td>
<td>1.5 bl/s</td>
<td>8 months</td>
<td>2116±326</td>
</tr>
<tr>
<td>swimming (n=8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum aerobic swimming (n=8)</td>
<td>up to 2.8-2.9</td>
<td>3 months</td>
<td>3954±329**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>on alternate days</td>
<td></td>
</tr>
</tbody>
</table>

* the weights of chinook salmon were between 650--700 g and the lengths were between 28--31 cm.
** denotes a significant difference (p<0.05) from control.
1 Two control groups were tested, one lasting 3 months, the other 8 months. There was no significant difference of $[^3\text{H}]$thymidine incorporation between two groups and they were pooled.
would never have been exhausted and swum a total distance of 3,766 km over an 8 month period.

C. Effect of burst swimming

Table 2 shows no significant difference (p<0.05) in $[^{3}\text{H}]$ thymidine incorporation into the coronary arterial smooth muscle as a result of enforced burst swimming three times a day for 3 days. These fish would have been exhausted 9 times, with a total swimming time of less than 90 min.
Table 2  Effect of Burst Swimming on $[^{3}\text{H}]$thymidine Incorporation into Coronary Arterial Smooth Muscle of Rainbow Trout*

<table>
<thead>
<tr>
<th>Fish Group</th>
<th>$[^{3}\text{H}]$thymidine (dpm/μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>2953±518</td>
</tr>
<tr>
<td>Burst Swimming (n=8)</td>
<td>1893±393</td>
</tr>
</tbody>
</table>

* Fish were chased to induce burst swimming until exhaustion (10 min), 3 times per day for 3 days. Tissues for culture were sampled on the third day after last swim.
IV. Discussion

IV.1 Methodology

The studies to develop and optimize growth conditions for coronary artery explants were an integral and necessary preliminary to the study of the effects of various factors on coronary vascular smooth muscle proliferation. Extensive and comprehensive experiments were performed to optimize the various growth conditions. From the experiments performed with various incubation times, it becomes clear that there was a substantial lag time before the explant began to incorporate $[^{3}\text{H}]$thymidine. The lag time before tissue outgrowth commences in culture systems different among different animal species and different tissues of the same animal. The lag time may be related to the time taken for phenotypic modulation to occur (i.e., the transition from contractile to synthetic vascular smooth muscle) and the rate of growth of explants after outgrowth has commenced (McMurry 1991). A small variability in lag time was found and it was thought to be a result of a variation in the speed of handling the tissue. Therefore, I standardized the time taken to process the tissue from animal to the explant stage.

A maximum $[^{3}\text{H}]$thymidine uptake occurred within 13 h of incubation with MHBS-1. This incubation time was considered sufficient since the types of experimental perturbation performed in Part One of my thesis would presumably have stimulated the vascular smooth muscle into the synthetic phenotype prior to culture. Indeed changes of 2-3 fold in uptake were observed. Therefore, the explant culture system was apparently sensitive
enough for the experimental design. In Part Two of my thesis, the experimental perturbations were different and so the medium was supplemented with serum and the incubation time was shifted to 48 h. This was because fatty acid metabolism and its effect on vascular smooth muscle cell mitosis were being stimulated during the culture.

Wolf and Quimby (1971) suggested MEM as a medium for routine fish cell and tissue culture. However, I found that MHBS-1 was better for coronary artery growth in salmonid species. The \([^3]H\)thymidine incorporation into coronary vascular smooth muscle in MHBS-1 was significantly higher than that in MEM.

A proper medium pH is necessary for good tissue culture growth. Most fish tissue cultures seem to fare well in the quite broad pH range of 7.2-7.8 (Wolf and Quimby 1971). This pH range is consistent with the plasma pH range of 7.2-7.8 in most fish (Heisler 1993). In my experiments I used a narrower range of pH. Although significant decrease in \([^3]H\)thymidine incorporation occurred at pH 7.3 and pH 7.9, differences were not detectable at pH 7.5 and pH 7.7. So I kept medium pH values as close to 7.6 as possible.

There is no effective buffer system in Hanks' balance salts. Therefore, to obtain pH stability during culture, I added sodium bicarbonate and TES as a two component buffer system for the medium. Sodium bicarbonate and TES are both commonly used buffers in tissue culture. Two chemical components were used in the buffer system because low concentrations of sodium bicarbonate or TES alone could not maintain pH values constant over the incubation period. By combining both chemical components, pH was
maintained at 7.6±0.1. Meanwhile, this two component system minimized the negative effect on tissue growth of a higher concentration of a single buffer.

The absolute level of [3H]thymidine incorporation did vary among the various control groups. Therefore, the results for each test were always compared with the control condition as a reference. Variation among controls likely reflected differences in the initial metabolic status of the coronary artery; this was due to factors such as seasonal temperature variation, ages of fish, species of fish, and other unknown factors which were not systematically investigated. For example, environmental temperature influences cardiac growth in fish. Cold-acclimation or cold-acclimatization increase the relative ventricular size in teleost fish (Kent and Prosser 1985; Tsukuda et al. 1985; Farrell 1987; Goolish 1987; Graham and Farrell 1989). Farrell et al. (1988) found that relative ventricular mass for 2+ and 3+ year-old rainbow trout was significantly lower in fish acclimated to 15°C than in fish acclimated to 5°C. Farrell et al. (1988) also found that fish age could influence ventricular growth. If the rate of cardiac myocyte growth and division can change in fish in response to environmental variables, it is possible that vascular smooth muscle of coronary artery does also.

The presence of [3H]thymidine in the nuclei of medial vascular smooth muscle was confirmed by limited autoradiographic studies. Several trials were performed before the correct emulsion/exposure time was established. It was evident that the majority of radiolabel was incorporated into the nuclei of medial vascular smooth muscle cells in AA-stimulated explants (most of the adventitia was stripped from the explant before incubation). I did not attempt systematic perturbations to ascertain whether not there were
regional differences in the incorporation of the radiolabel. Clearly, this is an area for future study. It would be especially interesting to determine whether an in vivo perturbation, e.g., mechanical injury, causes greater mitotic activity near the intima as would be expected. The outcome of my autoradiography is that a finding of increased [3H]thymidine incorporation into a coronary artery explant can be taken to confidently indicate an increase in the general level of medial vascular smooth muscle mitotic activity. Extrapolating this conclusion to the idea that increased vascular smooth muscle proliferative activity in explants reflects an increased potential for forming intimal lesions can only be made with a great degree of caution at this time. Certainly further studies are needed.

In summary, I was satisfied with the culture system for three reasons. First, vascular [3H]thymidine incorporation showed a significant time-dependent increase. Second, it was possible to modulate [3H]thymidine incorporation with a variety of physical and chemical, mechanical and environmental factors. Third, the radioautography observations clearly demonstrated [3H]thymidine incorporation into medial coronary artery smooth muscle nuclei. This [3H]thymidine incorporation into smooth muscle of the coronary artery could be quantitated by liquid scintillation counting of [3H]thymidine. In view of this, I am confident that the demonstrated effects of mechanical injury and repetitive, prolonged swimming to exhaustion on [3H]thymidine incorporation reflect an in vivo stimulation of medial smooth muscle proliferation in the coronary artery.
IV.2 Experimental interventions

A. Initiation of vascular smooth muscle proliferation \textit{in vivo}

\textit{a. Mechanical injury}

This study provides the first clear and convincing experimental evidence that gentle abrasion, which likely injured the vascular endothelium, increased mitotic activity in the medial vascular smooth muscle. The response to the mechanical injury was rapid. My studies identified a rapid peak in the mitotic activity at 48 h following the mechanical abrasion. There was no attempt made to identify whether or not mechanical abrasion stimulated vascular smooth muscle migration from the media to the intima.

Vascular injury is a widely accepted mechanism for initiating coronary disease in mammals (Ross and Glomset 1976, Ross 1986, 1993). Many experiments have already verified that injury to the endothelium of the coronary artery can lead to fast endothelial cell dysfunction. Capron and Bruneval (1989) observed a mitotic response of arteries to injury with a balloon catheter and found a strongly enhanced proliferating response of the aortic intima and media (media incorporation of $[^3]$H]thymidine in DNA) on day 2 after injury. This result shows that the mitotic response of the artery to mechanic injury is similar in both mammals and fish. After injury to the endothelium, the injured endothelial cells release the growth regulatory molecules and chemo-attractants. These regulatory molecules and chemo-attractants will induce migration and proliferation of smooth muscle which occurs in the early recurrence of stenosis after coronary angioplasty in

Although vascular injury may be an initiating factor in mammalian coronary vascular smooth muscle proliferation, a host of chemical signals are important sequelae to this event. In mammals, growth factors released from platelets and other blood cells play an important role in smooth muscle cell migration and proliferation. After the damage to the endothelial barrier, endothelial cells also release growth factors, e.g., thromboxane A₂ (TXA₂), prostaglandin F₂α (PGF₂α) (Hamberg et al. 1975; Moncada and Vane 1979) and basic fibroblast growth factor (bFGF) (Sato et al. 1991; Edelman et al. 1992; Reidy et al. 1992a, 1992b, 1993). Fish possess thrombocytes which are considered to have similar functions as mammalian platelets. Fish thrombocytes are known to be involved in blood coagulation and in prostaglandin (PG) and TXA₂ formation (Belamarich et al. 1966; Doolittle and Surgenor 1962; Woodward et al. 1981; Matsumoto 1988). If fish thrombocytes and endothelium synthesize TXA₂ and PGF₂α, these eicosanoids may also stimulate vascular smooth muscle proliferation. This suggestion is tested in Part Two of my thesis. Although it is not known if thrombocytes, monocytes and macrophages in fish release the growth factors, the physiological functions of these fish cells are known to be similar to those in mammals (Chiller et al. 1969, Ellis et al. 1974, 1976, Ferguson 1975). For example, the phagocytosis of macrophages (Mackmull and Michels 1932, Ellis and De Sousa 1974, Ferguson 1975) and monocytes (Ellis et al. 1976), the histochemical features of these blood cells and the basophilic cytoplasm
(Ellis et al. 1976), the same phagocytosis of macrophages, the surface antibodies of the cell membranes (Chiller et al. 1969, Ellis and De Sousa 1974) and the scavenging function of macrophages (Wolf and Quimby 1969, Weinreb and Weinreb 1969) are similar in both fish and mammals. Some of these are studied in Part Two.

My studies are limited in their support of the idea that vascular injury leads to coronary lesions in fish. However, an important first step, I observed a general stimulation of medial vascular smooth muscle proliferation in response to injury. Further studies are needed on possible stimulation of vascular smooth muscle migration, intimal vascular smooth muscle proliferation and lesion formation in response to mechanical abrasion in salmonids.

b. Swimming as an environmental stress

Saunders et al. (1992) hypothesized that coronary artery lesions may be linked to environmental stresses. The proposed linkage was an elevated ventral aortic blood pressure-induced distention of the ventricular outflow tract (bulbus arteriosus and ventral aorta) on which the coronary artery lies (as described in the introduction). This thesis provides the first, limited, experimental support for this idea by demonstrating a linkage between an environmental stress and medial vascular smooth muscle proliferation.

The environmental stress I selected was swimming because this relates to normal behaviors that fish might experience. My aim was to use four different swimming regimes to stimulate environmental stresses of varying
degrees. Because the cardiovascular response to these sorts of swimming challenges are well documented, especially by others in my laboratory, I did not measure blood pressure, cardiac output or blood catecholamine levels during any of my experiments. They were assumed to changed in the usual manner. The experiments provided qualitatively and quantitatively different types of swimming challenges. Significantly, the long term, low speed endurance trials had no effect on coronary vascular smooth muscle mitosis. Swimming at 0.5 bl/s, regardless of duration, is a relatively trivial swimming challenge for healthy salmonids. At this speed there would be little change in cardiac output, and certainly no significant increase in arterial blood pressure (Thorarensen 1994). Swimming at 1.5 bl/s represents more of an exercise challenge. The velocity of 1.5 bl/s was around 50-60% of the maximum prolonged swimming speed (Thorarensen 1994). Cardiac output would be elevated at this speed but not mean arterial blood pressure (Thorarensen 1994).

Of the two other training regimes, one involved burst swimming to fatigue nine times over 3 days. The other involved step increases in water velocity until fatigue was reached; the fish would have burst swam to fatigue at least 45 times over 90 days as well as swim to a velocity of 2-3 bl/s prior to fatigue. Since coronary vascular smooth muscle mitosis increased significantly in response to the longer exhaustive training regime, it appears that either more than 9 episodes of fatigue over 3 days are needed to stimulate coronary vascular smooth muscle mitosis, or swimming at a speed>1.5 bl/s is needed to produce the necessary stimuli for coronary vascular smooth muscle mitosis.
Thorarensen (1994) found that dorsal aortic blood pressure and cardiac output increased progressively as fish swum to speeds greater than 1.5 bl/s. Likewise, Kiceniuk and Jones (1977) found that cardiac output and ventral aortic blood pressure increased markedly with the maximum aerobic swimming speed. Thus, an important presumed cardiovascular difference between the fish swimming at 1.5 bl/s and the maximum aerobic swimming speed would be the elevated ventral aortic blood pressure and the higher cardiac output. Thus, if cardiovascular events are linked to coronary vascular smooth muscle mitosis, these two variables are good candidates for future studies.

Fish subjected to burst swimming alone would have had elevated arterial blood pressure and cardiac output, albeit less frequently and for a shorter duration. These cardiovascular changes were not associated with any stimulation of coronary vascular smooth muscle mitosis. It is not unusual for a stimulus to have frequency as well as intensity components to elicit a response. Thus, whereas mechanical injury was a sufficiently intense but brief stimulus to induce coronary vascular smooth muscle mitosis, and the maximum aerobic swimming regime was a sufficiently long and intense stimulus to induce coronary vascular smooth muscle mitosis, the burst exercise regime may not have been long enough. That the sham-operated group for the mechanical injury experiments also showed no significant increase in $[^{3}\text{H}]$thymidine incorporation is an important finding in this regard. This group of fish would have been stressed during the anaesthesia and surgery.
Even though the present data lend the first support to the idea that repeated and prolonged bouts of acute hypertension lead to coronary vascular smooth muscle mitosis, further experiments are needed to prove this linkage. Experiments involving ventral aortic banding for various periods of time may be fruitful in resolving both the issue of this linkage and the question of duration. In the interim, it would be unwise to eliminate factors other than the cardiovascular stimuli as being the stimulus for coronary vascular smooth muscle mitosis in salmonids. An involvement of oxLDL seems unlikely at this time since fat deposits and form cells are not found in fish coronary lesions. However, free radicals, e.g. superoxide radical, hydrogen peroxide and hydroxyl radical may be stimuli after artery wall injury and during exhaustive exercise. Recently, catecholamines have been demonstrated to be auto-oxidated to oxygen free radicals in mammals (Rump and Klaus 1994). Catecholamine concentration will greatly increase under stressful swimming conditions in fish (see Introduction); thus, the free radicals may correspondingly increase under stressful swimming. Stressful swimming may result in significant free radical production in fish.

Recent studies (Ward et al. 1983; Varani et al. 1985) show that free radicals, HO. or H2O2 can cause endothelial cell injury (Ward et al. 1983; Varani et al. 1985) in mammals. In a study of in vivo injury of rat pulmonary endothelial cells, Ward (Ward et al. 1983 1989) found the process of endothelial cell damage features the recruitment of neutrophils mediated by oxygen radicals. This injury of endothelial cells could be blocked by the presence of catalase. Singal et al. (1994) and Xiong et al. (1994) reported that free radicals could cause endothelial cell injury by oxidizing lipids and proteins as well as by causing strand breaks in nucleic acids in mammals. After injury by free
radicals, endothelial cells of coronary arteries can undergo a series of biochemical alterations and produce a variety of mitogens which stimulate vascular smooth muscle proliferation and finally cause coronary lesion formation. This process may be another factor for vascular smooth muscle proliferation and coronary lesion formation in salmonids.

IV. 3 Conclusion

Using [³H]thymidine as a rapid and sensitive in vitro assay of smooth muscle proliferation in fish coronary artery explants, the present results clearly indicate that medial vascular smooth muscle proliferation is initiated by gentle mechanical abrasion of the coronary artery and maximum aerobic swimming. These findings are the first preliminary support for Saunders and Farrell's hypothesis (1992) that mechanical injury linked to environmental stresses is an initiator of coronary vascular smooth muscle proliferation in salmonids. However, long-term experiments are needed to examine the influence of vascular injury related to environmental stress on the actual formation of coronary lesions and the influence of acute hypertension per se on coronary vascular smooth muscle mitosis. The involvement of vessel injury in vascular smooth muscle proliferation in salmon coronary arteries is also consistent with the widely accepted injury mechanism of atherosclerosis in mammals. It is also clear from my studies that not all environmental stresses lead to a significant stimulation of coronary medial vascular smooth muscle mitosis.
Part Two

Polyunsaturated fatty acids and eicosanoids as stimulatory factors in vascular smooth muscle proliferation
I Introduction

Coronary heart disease is the leading cause of death in human populations that consume diets rich in ω-6 polyunsaturated fatty acid (PUFA). Evidence has accumulated indicating that populations that frequently consume fish and fish oils have less coronary artery disease (Bang and Dyerberg 1972; Hirai 1980; Kagawa et al. 1990). The beneficial component of fish or fish oil appears to be the ω-3 PUFA, such as eicosapentaenoic acid (EPA).

Recent data suggest that fish oils may suppress the intimal proliferation of vascular smooth muscle cells in mammals (Thomas et al. 1983a; Morita et al. 1983). Smooth muscle cell proliferation has been recognized as a key event in the initiation and progression of atherosclerosis, and a well documented reaction to coronary artery injury (Ross and Glomset 1976; Reidy 1985; Ross 1986; Nilsson 1986; Schwartz 1986; Morisaki et al. 1988a; Jackson et al. 1988; Stary 1989). If this is the case, it is difficult to explain why lesions, consisting almost entirely of intimal vascular smooth muscle cells, develop in coronary arteries of salmon when these fish have naturally high levels of ω-3 PUFA. By investigating the effects of PUFA, PUFA metabolites and metabolic pattern on vascular smooth muscle proliferation of coronary artery explant from fish, I expect to provide insights into why coronary lesions develop in fish despite high levels of ω-3 PUFAs.
I.1 PUFAs and their metabolites

A. PUFAs: chemical structures and metabolism

Most naturally occurring fatty acids are essentially long hydrocarbon chains with a carboxylic acid group at one end. The fatty acids are characterized by the length of the chain of carbon atoms, the number of double bonds, and the position and orientation of the double bonds. Fig. 15 shows the molecular structures of two important PUFAs: eicosapentaenoic acid (EPA) (it has 20 carbons and 5 double bonds; 20:5) and arachidonic acid (AA) (it has 20 carbons and 4 double bonds; 20:4). Because the last double bond of EPA is located at the third carbon position counted from the methyl terminator, it belongs to the ω-3 PUFA family, i.e., 20:5 ω-3. Similarly, the last double bond of AA is located at the sixth carbon position counted from the methyl terminator, therefore it belongs to the ω-6 PUFA family, i.e., 20:4 ω-6.

PUFAs are a major component of the PL fatty acids in cell membranes. The metabolic fate of fatty acids after assimilation from the digestive tract is varied. Their involvement in the formation of PL is as follows. All fatty acids are transported in the blood in a form associated with lipoproteins or plasma proteins. At a target tissue, the triacylglycerols in lipoproteins are cleaved by lipoprotein lipase, which lies at the end of a polysaccharide chain on the cell surface, yielding glycerol and free fatty acids. The dissociated fatty acids enter the cell by passive diffusion, where they can be incorporated into PLs. The PLs form an integral part of the tissue membranes. Gunstore et al. (1978) reported that PUFAs account for up to 58% of the fatty acids
Fig. 15. Molecule structures of EPA and AA.
Polyunsaturated Fatty Acids

Eicosapentaenoic acid (EPA), 20:5\(\omega-3\)

Arachidonic acid (AA), 20:4 \(\omega-6\)
present in total PLs in fish. The PUFA in PL is esterified usually at the second or occasionally the first carbon position of a glyceryl. Different PUFA families are competitively esterified into the membrane PL according to their substrate concentrations. In this way, dietary PUFA composition greatly influences PUFA composition of PLs in tissue membranes. For example, it is known that if the ω-3 PUFA concentration in diet is higher than ω-6 PUFA, a higher ratio of ω-3 PUFA to ω-6 PUFA will result in tissue PL of humans and fish (Dyerberg and Bang 1979; Watanabe 1974; Croft et al. 1988; Bell et al. 1985, 1991, 1992; Schmidt et al. 1993).

PUFAs are integral components of phospholipids; therefore, any stimulus or insult which degrades cellular phospholipids will release PUFAs. Two factors stimulate PUFA release from PL: chemical (e.g., hormonal) stimuli, and mechanical stimuli (Hsueh et al. 1977, 1979). That mechanical stimulus is associated with the release of PUFAs is evident from studies of conditions associated with cell damage (e.g., ischemia, cell death, and mechanical trauma to the vessels, (Cenedella et al. 1975; Bazan and Rakowski 1970; Piper and Jane 1971). As Piper and Jane (1971) concluded from their studies of lungs, "the common thread which runs through the different ways of causing PUFA release is the disturbance of the cell membrane. This may range from simple distortion to stretch to rupture".

Fatty acids can be released from PLs of cell membranes through two pathways (Gerrard 1985). The first involves the phospholipase A₂ pathway which lies within a dense tubular system. Phospholipase A₂ cleaves the fatty acid in the two-position of the PL. Fatty acids in the sn-2 position are usually unsaturated (Gerrard 1985). The second involves phospholipase C
pathway, which is generally presumed to be a cytoplasmic event because phospholipase C is a water soluble enzyme (phospholipase C is thought by some to be loosely associated with the plasma-membrane and possibly within the dense tubular system). Phospholipase C hydrolyzes PLs to produce diacylglyceriole (DAG) and phosphorylated alcohol (i.e., inositol, choline, ethanolamine or serine phosphate). DAG is the substrate for a DAG lipase located in the dense tubular system; this enzyme first cleaves the fatty acid in the sn-1 position and then hydrolyzes the PUFA from the sn-2 position (Bell et al. 1979).

Once released, PUFAs are rapidly metabolized into oxygenated products by two distinct pathways involving either lipoxygenase or cyclooxygenase. The lipoxygenase enzyme appears to be cytosolic, but might be loosely membrane bound (Gerrard 1985b). The end products of these two pathways differ greatly according to the initial substrate (EPA or AA) and other secondary enzymes active in the cells. In one pathway, lipoxygenase (Fig. 16a, 16b) attacks various double bonds to form several unstable compounds known as hydroperoxy fatty acids and leukotrienes (Borgeat and Samuelsson 1979a, 1979b). The synthesis of AA-derived leukotriene (LTs) by 5-lipoxygenase involves an initial oxidation of arachidonic acid at carbon 5 to yield 5-hydroperoxyeicosatetraenoic acid (5-HPETE) followed by the subsequent conversion of 5-HPETE to 4-series leukotriene A₄ (LTA₄). LTA₄ is subsequently enzymatically converted to the leukotriene C₄ (LTC₄) or leukotriene E₄ (LTE₄). In contrast, with EPA substrate the 5-lipoxygenase conversion products will be 5-series leukotriene (LT).
Fig. 16a. Arachidonic acid-derived eicosanoids synthesis pathway. The abbreviations in this figure represent the compounds as below: 5-HPETE (5(S)-hydroperoxyeicosa-6E,8Z,11Z,14Z-tetraenoic acid), LTA₄ (leukotriene A₄), LTB₄ (leukotriene B₄), LTD₄ (leukotriene D₄), LTE₄ (leukotriene E₄), LTF₄ (leukotriene F₄), PGG₂ (prostaglandin G₂), PGH₂ (prostaglandin H₂), PGD₂ (prostaglandin D₂), PGE₂ (prostaglandin E₂), PGF₂α (prostaglandin F₂α), PGI₂ (prostaglandin I₂), TXA₂ (thromboxane A₂).
The Omega-6 Family

Dietary Fatty Acids

Linoleic Acid (18:2 ω-6)

γ-Linolenic Acid (18:3 ω-6)

Dihomo γ-Linolenic Acid (20:3 ω-6)

Arachidonic Acid (20:4 ω-6)

Lipoxygenase

5-HPETE

LTA₄

LTC₄

LTD₄

LTE₄

LTF₄

Cyclooxygenase

PGG₂

PGH₂

PGI₂

PGE₂

PGD₂

PGF₂

TXA₂
Fig. 16b. Eicosapentaenoic acid-derived eicosanoids synthesis pathway. The abbreviations in this figure represent the compounds as below: 5-HPEPE (5(S)-Hydroperoxyeicosa-6E,8Z,11Z,14Z,17Z-pentaenoic acid), LTA₅ (leukotriene A₅), LTB₅ (leukotriene B₅), LTD₅ (leukotriene D₅), LTE₅ (leukotriene E₅), LTF₅ (leukotriene F₅), PGG₃ (prostaglandin G₃), PGH₃ (prostaglandin H₃), PGD₃ (prostaglandin D₃), PGE₃ (prostaglandin E₃), PGF₃α (prostaglandin F₃α), PGI₃ (prostaglandin I₃), TXA₃ (thromboxane A₃).
The Omega-3 Family

Dietary Fatty Acids

α-Linolenic Acid (18:3 ω-3)

Eicosapentanoic Acid (20:5 ω-3)

Lipoxygenase

5-HPEPE

LTA$_5$

LTC$_5$

LTD$_5$

LTE$_5$

Cyclooxygenase

PGH$_3$

PGG$_3$

PGE$_3$

PGF$_3\alpha$

PGD$_3$

TXA$_3$
In another pathway (Fig. 16a, 16b), cyclooxygenase forms an unstable cyclic endoperoxide, prostaglandin G2 (PGG2) with AA as a substrate. This substance is, in turn, converted to another unstable endoperoxide, prostaglandin H2 (PGH2). PGH2 is broken down enzymatically to the stable prostaglandin E2 (PGE2), prostaglandin F2α (PGF2α), and prostaglandin D2 (PGD2), a 17-carbon hydroxy acid (12-hydroxy-5, 8, 10-heptadecatrienoic acid) and malondialdehyde. The prostaglandin endoperoxides are also transformed by other enzymes into prostacyclin (PGI2) and TXA2, both of which are chemically unstable, breaking down to 6-keto-PGF1α and TXB2. If the substrate is EPA the cyclooxygenase conversion products will be 3-series PG and TX.

The above eicosanoid pathways occur widely in animals, including human and fish (Granstrom et al. 1985; Bell et al. 1991a; Henderson and Tocher 1987). The study of lipoxygenase activity from trout gills indicated that the lipoxygenase inhibitors, SnCl2 and esculetin, could strongly inhibit the activity of lipoxygenase (German et al. 1986). A study on the effect of PG on ovulation in teleosts by Kapur and Toor (1979) and by Slacey and Goetz (1982) showed that indomethacin, a potent inhibitor of PG formation, blocks both PG synthesis and ovulation. The inhibitory effect of indomethacin on PG synthetic pathway in fish suggested a similar metabolic pathway for PG formation as that in mammals (Espey 1980).
B. Cardiovascular effects of PUFAs and their metabolites

Populations consuming more ω-3 PUFA from fish and sea mammals have less coronary heart disease (Dyerberg et al. 1978; Dyerberg and Bang 1979; Kromhout 1985). This conclusion was originally made from the studies with Eskimos. Diets containing ω-3 PUFA have also been found to reduce the severity of experimental cerebral (Black et al. 1979) and myocardial (Culp et al. 1980) infarction in rats.

Diets rich in ω-3 PUFAs are known to have at least five potential anti-atherogenic effects in humans: 1) A lowering effect on plasma lipid and lipoprotein levels (Herold and Kinsella 1986); 2) A decreased potential for platelet aggregation (Goodnight et al. 1982; Harris et al. 1983); 3) An inhibition of intimal hyperplasia in canine autologous vein grafts (Landymore et al. 1986; Cahill et al. 1988); 4) A potential reduction in the intensity of the vascular inflammatory response (Lee et al. 1985; Fisher et al. 1986); and 5) An inhibition of the production of mitogens that affect SMCs (Morita 1983; Karmazyn 1989; Lands 1992).

Central to my studies are the effects of ω-3 PUFA and ω-6 PUFA on vascular smooth muscle. An inhibitory effect of ω-3 PUFA on ω-6 PUFA has been verified in mammalian atherosclerosis (Morisaki et al. 1982, 1983; Karanazyn 1989; Lands 1992; Schmidi et al. 1993). In contrast to ω-3 PUFA, ω-6 PUFA stimulates atherosclerosis development in mammals (Moncada and Vane 1979). For example, ω-6 PUFA promotes platelet aggregation (Herold and Kinsella 1986) and thrombosis formation (Lands 1992), stimulates smooth muscle cell proliferation (Morisaki et al. 1983; Pomerantz
and Hajjar 1989), and causes vasoconstriction (Herold and Kinsella 1986). Thus, ω-3 PUFA rich diets appear to inhibit the atherogenic properties of ω-6 PUFA. There are two possible mechanisms to explain the inhibitory effects of EPA (ω-3 PUFA) on AA (ω-6 PUFA). One possibility is that EPA feeding may cause a shift from prostacyclin (PGI2) and TXA2 production to PGI3 and TXA3 production (Raz et al. 1977). EPA is metabolized to TXA3 to replace TXA2, a metabolic product of AA, and then plays a feedback inhibitory role on the production of TXA2 (see also Figs 16a and 16b). The second possible mechanism is that the EPA itself may competitively block the oxidation of AA in cells. In EPA-treated cells, Morita et al. (1983) found that the content of AA in phospholipid fractions decreases, while the content of EPA increases. So via the competitive incorporation into PL, the amount of cellular free-AA released from PL is reduced in EPA-treated cells.

It is generally believed that the effects of PUFAs on coronary arterial lesions are linked to their metabolites, the eicosanoids. The major eicosanoids already implicated as potent mitogens in the regulation of vascular smooth muscle cell proliferation and as chemo-attractants in platelet aggregation are the counteracting AA-derived eicosanoids, TXA2 and PGI2 (Samuelsson et al. 1978; Moncade 1982; Akopov et al. 1988; Morisak et al. 1988a, 1988b). Indeed, the atherosclerotic process is associated with diminished PGI2 and elevated TXA2 plasma concentrations (Simmet and Pesker 1986). TXA2 is a potent mitogen in smooth muscle cell proliferation (Vane 1978; Samuelsson et al. 1978; Moncade 1982; Akopov et al. 1988; Gerred et al. 1989), promotes foam cell lesion formation (Skrinska et al. 1988), and stimulates platelet aggregation (Moncada et al. 1979). In contrast, PGI2 is an antagonist of TXA2 which inhibits smooth muscle cell proliferation (Morisaki et al. 1987,
1988) and platelet aggregation (Moncada et al. 1979). Besides PGI₂, the EPA metabolites TXA₃ and PGI₃ are also antagonists of TXA₂. TXA₃ lacks the deleterious biological activity of TXA₂ on vascular smooth muscle and platelet aggregation. PGI₃ is also antiaggregatory. When EPA in the diet displaces AA, it results in the in vivo formation of TXA₃ and PGI₃. In addition, the lipoxygenase products, the AA-derived eicosanoids, TXA₂, produce deleterious effects including coronary constriction and cardiovascular depression (Gerred et al. 1989). Dietary intake of AA produces LTB₄ and LTD₄, which are powerful mitotic and chemotactic compounds produced by polymorphonuclear leucocytes, macrophages, neutrophils and the arterial wall (Faggiotto 1986; Lewis et al. 1986). When EPA replaces AA in the diet, LTB₅ and LTD₅ are synthesized and have a markedly lower potency than LTB₄ and LTD₅ with respect to their mitotic and chemotactic roles. This decreases the proliferative and inflammatory response at the site of injury to the vessel wall.

C. PUFAs and their metabolites in fish

a. PUFA constitution

In general, freshwater fish are characterized by high ω-6 PUFA content in their tissues whereas marine fish and fishes that have a marine phase of their life cycle (such as salmonids) are characterized by low ω-6 and high ω-3 PUFA (Ackman and Takeuchi 1986; Gong and Farrell 1990). This difference arises primarily from the dietary quality of environments in which fish live.
The fatty acid composition of tissue lipids in fish are markedly influenced by the pattern of fatty acids in their dietary lipid, as illustrated by many studies on the PUFA requirements of salmonids (Castell et al. 1972; Yu and Sinnhuber 1972; Watenabe et al. 1974; Takeuchi and Watenabe 1982; Gong and Farrell 1990). The fatty acid profile of lipids in wild fish reflects the availability of fatty acids in the aquatic food chain. Algae feature prominently in the diet of the early life stages of some freshwater fish (Henderson and Tocher 1987). In general, the lipids of freshwater algae contain higher levels of ω-6 PUFA, e.g., 18:2 (ω-6) and 20:4 (ω-6). Aquatic insects, larval and nymphs, are a major food source for freshwater fish, particularly salmonids (Henderson and Tocher 1987). These aquatic insects contain high levels of 18:2 (ω-6), 20:4 (ω-6) and 18:3 (ω-3). The lipids of predatory fish preying on aquatic insects can be expected to be influenced by the fatty acid composition of their prey. On the other hand, the phytoplankton and zooplankton of the marine food chain contain lipids in which ω-3 PUFA, e.g., 18:3 (ω-3), 20:5 (ω-3) and 22:6 (ω-3) predominate and which have low levels of ω-6 PUFA (Henderson and Tocher 1987).

Water temperature can also influence fatty acid composition. Cold water can increase the proportion of ω-3 series of PUFA in trout (Roots and Johnston 1968; Hazel and Prosser 1979). Fish are poikilotherms in which the cell membranes encounter various and often low environmental temperatures. In such systems the temperatures encountered by the membranes are often low enough to be around the phase transition of some of the phospholipid species. Some phase separation is likely to occur and, in any area of gel-phase lipid, the functions of membrane proteins and the permeability properties of the membrane are likely to be impaired. It is important that
this situation is countered and there is much evidence of changes in lipid composition directed to this end. PUFA content of phospholipids increases on adaptation to lowered environmental temperatures, especially ω-3 PUFA is favoured over ω-6 PUFA (Bell et al. 1986). Hazel and Prosser (1979) and Sellner and Hazel (1982) found that hepatocytes isolated from the livers of rainbow trout acclimated to 5°C had higher proportions of PUFA, particularly the ω-3 series, in their total lipid than cells from warm (20°C) acclimated fish. Other workers have also noted that the main change in fatty acid composition at low temperature is an increase in ω-3 PUFA in the gills of eel (Thomson et al. 1977) and carp liver (Farkas 1984). An advantage of the shift to ω-3 PUFA is related to the lower melting point (m.p.) of ω-3 PUFA compared with ω-6 PUFA. As a result, ω-3 PUFA can maintain membrane fluidity better than ω-6 PUFA at lower temperatures. In general, an increase in unsaturation is considered to increase membrane fluidity. However, the m.p. depends more on the position of the double bonds than on the number of double bonds. For example, the m.p. of AA (20:4 ω-6) is -49.5°C, whereas the m.p. of EPA (20:5 ω-3) is -54.4°C. The nearer the double bonds are to the center of the chain the lower the m.p. is (Bell et al. 1986).

Salinity can also affect PUFA composition in fish tissues. Leray et al. (1984) found that the changes of fatty acid patterns of tissue lipids were associated with sea water adaptation. The most significant alteration was observed in the fatty acid composition of intestinal membranes of trout transferred from fresh to sea water, occurring in the major phospholipid, phosphatidylcholine, in which the proportion of total ω-3 PUFA increased from 17.7% to 33.4% within one day of transfer. Correspondingly, the level of ω-6 PUFA fell from 8.3% to 6.0%. In the guppy, Poecilia reticulate, Daikoku et al. (1982) also
found an increase in ω-3 PUFA on adaptation to seawater but, in contrast to trout, they found the proportions of phosphatidylcholine and phosphatidylserine changed in various tissues.

The above findings clearly show that the high levels of ω-3 PUFA in marine fishes, while dictated primarily by their high levels of ω-3 PUFA of feed, are also influenced by their physical environment. Kanazawa et al. (1982) performed an incorporation study of dietary radioactive EPA using larval ayu; they found a high incorporation of EPA into many tissues. The authors concluded that this fatty acid was likely to be utilized as a constituent of cellular membranes for growth rather than being oxidized for energy and metabolites. Based on these data, Kanazawa (1982) postulated that relatively large amounts of exogenous EPA may be necessary for fish growing at a rapid rate.

*b. Features of PUFAs composition in PL and their metabolism*

Eicosanoids occur throughout the animal kingdom from mammals to invertebrates (Bell et al. 1986). They have been found in a marine coral (Weinheimer and Spraggins 1969) and a marine algae (Fusetani and Hashimoto 1984). Eicosanoids are known to occur in fish tissues compared to mammals but not much work has been done on their synthesis and physiological roles in fish. Prostaglandins can be synthesized by all fish tissues so far studied, including gills, kidney, spleen, intestine, stomach, liver, heart, brain, skin, ovarian fluid, testes and blood thrombocytes (Kayama et al. 1986; Nomura et al. 1973, 1976; Ogata et al. 1978). Although ocean-going fish tissue lipids contain high levels of ω-3 PUFA, there may be a
special mechanism of preferential incorporation of ω-6 series of PUFA (AA) into their PL for eicosanoid formation, especially into phosphatidylinositol (PI) (Bell et al. 1985; Ackman and Takeuch 1986; Henderson and Tocher 1987). PI is thought to be one of the main suppliers of PUFA for eicosanoid formation in mammals (Marshall et al. 1981; Irvine 1982). Besides providing AA for synthesis of eicosanoids, PI is also involved in the formation of the polyphosphoinositides and subsequently the formation of the hormonal second messengers inositol triphosphate and diacylglycerol (Berridge 1984). The polyphosphoinositides, inositol triphosphate and diacylglycerol are important mitogenic signals in cellular proliferation in mammals (Berridge 1984).

In fish, PI is a main PUFA supplier for eicosanoid formation (Bell et al. 1986). Arachidonic acid appears to be a major precursor for eicosanoid production in fish gills, skin, liver, kidney, stomach, and intestines (Anderson et al. 1981; Bandyopadhya et al. 1982; Henderson 1985; Bell et al. 1986). Apart from providing PUFA, PI metabolites (inositol triphosphate, diacylglycerol and polyphosphoinositide) in fish may also play an important mitogenic role in the cellular proliferation, as found in mammals (Bell et al. 1986).

Although conversion of ω-3 PUFA and ω-6 PUFA into eicosanoids can occur in both mammals and fish, the conversion rate of both PUFA families appears different. Whereas in mammals the PUFA conversion is competitive and related to the amount of parent PUFA, this is apparently not the case in fish.
In humans eicosanoid formation is quantitatively related to the amount of the parent fatty acid family ratio in the cellular PL. In mammals, the conversion rates of cellular EPA and AA to eicosanoids are similar if the both PUFA substrate concentrations are same. Strasser et al. (1985) found that the conversion rates of cellular EPA and AA to LTB₅ and LTB₄ were similar from peripheral human neutrophils after dietary supplementation with the same amount of EPA or AA diet to volunteers. However, one family of PUFA can be competitively incorporated into PL, hence blocking conversion of another family of PUFA. For example, at high concentrations of exogenous EPA, the formation of AA-derived PG was suppressed (Strasser et al. 1985). Feeding dietary supplements of marine oils rich in ω-3 PUFA to mammals increase the content of ω-3 PUFA in cellular phospholipids (Iritani and Fujikana 1982) and decrease the formation of PGE₂ from AA (Schoene et al. 1981). Fischer and Weber (1984) also found that the formation of TXA₂ was diminished by dietary EPA in humans. All these effects of ω-3 PUFA are due to a reduced availability of AA substrates from PL. Thus, the conversion ratio of ω-3 and ω-6 PUFA in PL of mammals reflects the ratio of available EPA to AA.

The conversion ratio of ω-3 and ω-6 PUFA in fish does not always reflect the ratio of available EPA and AA in PL. While there are limited data, to date, AA is the preferred substrate for the cyclooxygenase and lipoxygenase in spite of the preponderance of ω-3 PUFA, especially EPA, in marine fish lipid (Anderson et al. 1981; Henderson et al. 1985, 1987). When AA and EPA were added individually at the same concentration, Anderson et al. (1981) found that microsomes of plaice skin converted 2% of AA but only 0.3% of EPA to PGE₂ and PGE₃, respectively. Henderson et al. (1985) found that despite
the abundance of EPA in the tissue lipid of marine fish, the conversion of $^{14}\text{C}-\text{AA}$ had a two-fold greater rate of conversion to prostaglandin material than $^{14}\text{C}-\text{EPA}$ when these fatty acids were presented at the same concentration to homogenates of turbot gill tissue. Through comparative studies of exogenously added AA and EPA, Tocher and Sargent (1986) found that AA was the preferred substrate for the 5-lipoxygenase metabolism in peripheral blood neutrophils from a marine teleost, the plaice. Thus, a special pattern of preferential metabolic utilization and conversion of AA in fish tissue may constitute one of the important factors in the development and promotion of coronary arterial lesions in fish.

c. Known functions of eicosanoids in fish

In fish, eicosanoids play important roles on physiological activities. The identification of prostaglandins in testes and ovaries of fish (Goetz 1980; Nomura et al. 1973) implied a role in fish reproduction. Intraperitoneal injection of PGE$_1$ or PGE$_2$ into female catfish (Heteropneustes fossilis) induced ovulation (Singh et al. 1976), whereas the cyclooxygenase inhibitor, indomethacin, blocked ovulation in carp (Kapur and Toor 1979). Clomiphene citrate, which induces gonadotrophin release, restored ovulation in indomethacin-treated carp (Breton et al. 1975), suggesting that indomethacin blocks pre-ovulatory gonadotrophin release. Prostaglandins are probably also required more directly for ovulation (Stacey et al. 1982). In addition, prostaglandins may act on the brain to elicit behavioral changes, such as spawning activities (Stacey 1976).
Piomelli (1985) demonstrated that leukotrienes from eel gills had the same biological effects on mammalian lung tissue as mammalian LTC₄. It is possible that LTC₄ may play a role in modulating gill function at a local level. Bell et al. (1985) and Praag et al. (1987) found that both cyclooxygenase and lipoxygenase metabolites of AA were involved in the regulation of active transport across the gills and opercular epithelium. They observed that chloride transport was stimulated by LTC₄, LTD₄, and LTE₄ and inhibited by PGE₂. LTB₄ was found to have a chemotactic activity for plaice leukocytes (Tocher and Sargent et al. 1987), suggesting a similar function for this derivative in both fish and mammals. The identification of TXB₂ in rainbow trout thrombocytes (Kayama et al. 1986) suggested that the control of thrombocyte aggregation and blood clotting in fish may involve a TXB₂/PGI₂ balance, as is known for mammals.

Bell et al. (1991) found that Atlantic salmon fed ω-6 PUFA-rich (and ω-3 PUFA depleted) diets developed heart lesions that caused a thinning of the ventricular wall and muscle necrosis. The fish were also susceptible to a transportation-induced stress and had high mortality compared to ω-3 PUFA-rich fed fish. The authors concluded that a diet with a low ω-3/ω-6 PUFA ratio caused changes in fatty acid metabolism that caused the heart lesions and susceptibility to stress.

In summary salmonid tissues contain an abundance of ω-3 PUFA, but the fish still develop coronary arterial lesions. Although information on the physiological functions of both cyclooxygenase and lipoxygenase metabolites of AA in fish are far from as detailed as that in mammals, recent data suggest that these eicosanoids, when present, can perform similar functions.
in fish as they do in mammals. This conclusion provides a basis for examining the potential effects of AA metabolites on coronary arterial smooth muscle proliferation in fish. To date, there are no reports concerning the effects of PUFAs and their metabolites on coronary vascular smooth muscle proliferation in fish.

The inhibitory physiological roles of PUFAs and their metabolites on coronary arterial lesions in mammals provide the theoretical basis for my studies on the relationship between coronary lesion initiation and the effect of PUFAs and their metabolites in fish. Because initiation of vascular smooth muscle proliferation is a central feature of atherosclerosis in both mammals and fish, and a well-documented reaction to coronary artery injury, the effects of PUFAs and their metabolites on the vascular smooth muscle proliferation are the focus of Part Two of my thesis. The following research objectives were planned to address above concerns.

I.2 Objectives

1. To perform in vitro dose-response studies with coronary artery explants (a) to examine the hypothesis that ω-3 PUFA (EPA) inhibits medial vascular smooth muscle proliferation, and (b) ω-6 PUFA (AA) metabolites induce medial vascular smooth muscle proliferation. These are the first studies of the mitogenic effect of PUFA in fish.

2. To perform biochemical analyses of the fatty acid composition of phospholipids in active PUFA conversion tissue, gills, to provide insight into the pattern of metabolism of PUFA in fish.
II Materials and Methods

II.1 *In vitro* dose-response manipulations of $[^{3}\text{H}]$thymidine incorporation into coronary arterial explants

The explant preparation and incubation conditions are fully described in Part one of my thesis. All incubations were for 48 h after which dpm and DNA were measured. One explant was removed from each fish and three replicate explants were used for each concentration tested of each PUFA and eicosanoid metabolites.

A. Effects of Pure PUFAs

a. *Rationale*

In order to understand the exclusive effects on vascular smooth muscle proliferation of either $\omega$-3 PUFA or $\omega$-6 PUFA alone or in combination, $\omega$-3 and $\omega$-6 PUFA were incubated with coronary explant for 48 h.

b. *Experimental design and protocol*

EPA (eicosapentaenoic acid, 20:5 $\omega$-3), AA (arachidonic acid, 20:4 $\omega$-6) and ETA (eicosatrienoic acid, 20:3 $\omega$-6) (99% pure, Sigma Chemical Co.) were all dissolved as needed in 95% ethanol which had been saturated with $\text{N}_2$ and chilled on ice in advance. The purity of the PUFAs were examined with gas chromatography. The vials containing the diluted PUFAs were stored at -20°C in a black, airtight container for no longer than two days before use.
An initial range-finding experiment was performed by R. Townley (R. Townley Honors Thesis 1992) under my supervision to determine the PUFA concentration needed for effective incorporation into the vascular smooth muscle cell metabolic pathways. He found that PUFA concentrations of 20, 40 and 100 μM could stimulate coronary explant growth to different extents (see Appendix I). Therefore, I used concentrations of 20, 60, 80, and 120 μM in my experiments.

Subsequent experiments on the interactive effects of EPA or ETA and AA on [3H]thymidine incorporation into coronary arterial smooth muscle used 20 μM AA in combination with either EPA concentrations of 20, 50, 100, and 200 μM, or ETA concentrations of 10, 20, 50, 80, and 120 μM. AA at 20 μM was selected because this concentration of AA had the greatest stimulatory effect on vascular smooth muscle mitosis. All PUFAs were added into the culture medium at 0 h. An equal amount of 95% ethanol was added to control cultures.

B. Effects of arachidonic acid (AA)-derived eicosanoids

a. Rationale

The focus of this experiment was on major AA-derived eicosanoids, i.e., thromboxane A2 (TXA2), prostaglandin I2 (PGI2), prostaglandin F2α (PGF2α) and leukotriene C4 (LTC4), that are known to regulate vascular smooth muscle cell proliferation or migration in mammals. Among them, TXA2 stimulates and PGI2 inhibits vascular smooth muscle proliferation in
mammals. PGF$_2\alpha$ is inhibitory at low concentrations and stimulatory at high concentrations. LTC$_4$ is also a stimulatory factor and a chemotactant in mammals. I examined the effects of these four eicosanoids on vascular smooth muscle mitosis in fish.

b. Experimental design and protocol

All eicosanoids, PGF$_2\alpha$, LTC$_4$, and eicosanoid analogues, U-46619 (a TXA$_2$ analogue) and carbacyclin (a PGI$_2$ analogue) (Biomol Research Laboratories Inc., Ont.), were prepared just before use. Eicosanoids and analogues used in this experiment were all dissolved in 95% ethanol which had been saturated with N$_2$ and chilled on ice in advance. Transfer and removal of samples were carried out on ice. All prepared chemicals were kept in dark colored vials. Stable analogues of TXA$_2$ (U-46619) and PGI$_2$ (carbacyclin) were used in this experiment because of the unstable nature of the parent compounds under physiological conditions. For example, the half life of TXA$_2$ under physiological condition is half a minute.

The concentrations tested were based on previous studies with mammalian vessels. Morisaki et al. (1988a, 1988b) and Avopov et al. (1988), working with cultures of smooth muscle cells from intima of rabbit aorta, used U-46619 and carbacyclin concentrations from 0-600 ng/ml as exogenous chemical to observe their effects on DNA synthesis in smooth muscle cells. Therefore, concentrations of 100 (0.28 nM), 400 (1.14 nM), 700 (2.00 nM), and 1000 (2.85 nM) ng/ml were used for U-46619 (MW=350.5) and carbacyclin (MW=350.5), and 100 (0.16 nM), 400 (0.64 nM), 700 (1.12 nM) and 1000 (1.60 nM) were used for LTC$_4$ (MW=625.8) in my experiments. Concentrations of
30 (0.08 nM), 60 (0.17 nM), 90 (0.25 nM) and 120 (0.34 nM) ng/ml were used for observing the effect of PGF$_2$$_\alpha$ (MW=354.5). Eicosanoids and their analogues were all added into culture media at 0 h. An equal amount of 95% ethanol was added to control cultures. The final concentration of ethanol in the culture medium was 0.6%.

II.2 Biochemical analyses for PUFA composition in phospholipid (PL) of gills

A. Rationale

As suggested in the introduction (see Part two I.1.C.b), there might be a mechanism of preferential incorporation of $\omega$-6 PUFA into eicosanoid synthesizing PL and tissues from salmonids, despite the high $\omega$-3 PUFA levels. If this suggestion is true, then one would expect AA to predominate in PLs of fish. To verify this prediction, an analysis of PUFAs from PI and other PLs of gill tissue was performed. The major precursors of eicosanoids in fish are AA and EPA. Saturated fatty acids are the major component in the deposited lipids of fibrous plaque in mammals and play an important role in atherosclerosis formation in mammals. However, in fish there are no lipid deposits in coronary artery lesions (Massey 1984; Farrell et al. 1986, 1990). Thus, saturated fatty acids are not discussed here. PI was chosen in the present experiment because it is a major supplier of PUFA for eicosanoid formation in fish (Bell et al. 1986) and it plays a pivotal role in the transduction of hormone signals (Bell and Sargent 1985). Gill tissue was selected because it is the most active fish tissue for eicosanoid synthesis (Bell et al. 1986). Juvenile fish (parr) in fresh water were used because of the very low incidence of coronary artery lesions at that age. A comparison was
made with mature fish known to have coronary artery lesions (Farrell et al. 1991).

B. Experimental design and protocol

a. Preparation of gill tissue

Five groups of parr, each group consisting of a pool of four individual parr, and five individual mature coho salmon samples were compared. Gill arches were excised and rinsed in ice-cold saline (0.7% NaCl) to remove adhering blood. Approximately 1 g of wet primary gill filaments was used for the 10 determinations (5 parr and 5 mature fish).

b. Experimental procedure: lipid extraction and analysis

Each gill sample was homogenized in 10 volumes of chloroform:methanol (2:1 by volume) for extraction of lipids according to the method of Blight and Dyer (1959). Homogenates of the tissue were centrifuged at 8,000 g and the solvents evaporated under N₂. The polar lipids were separated with a large amount of neutral lipid by thin layer chromatography (TLC) on silica gel G with fluorescence (Merck, Co.) using hexane:diethyl ether:glacial acetic acid (80:20:2 v/v/v) as the developing solvent. Polar lipids were eluted from the origin using chloroform:methanol:water (5:5:1 v/v/v) and concentrated under a stream of nitrogen. Separation of PL fractions from polar PL was performed by two dimensional TLC on silica gel G with fluorescence using chloroform:methanol:water:ammonia (130:70:8:0.5 v/v/v/v) in the first dimension and chloroform:acetone:methanol:glacial acetic acid:water
(10:4:2:2:1 v/v/v/v/v) in the second dimension (Parsons and Patton 1969). The individual phospholipid zones were visualized under UV light, scraped from the plates and eluted with chloroform:methanol (1:1 v/v). The solvent was removed under a stream of nitrogen.

The phospholipids were transmethylated in 1.5 ml of boron trifluoride (14%)-methanol (Sigma Chemical Co.) at 70°C for one hour under nitrogen (Sheridan et al. 1985). After adding 1 ml of distilled water (Tocher et al. 1989), the methyl esters were extracted into 3 ml of hexane. The extracts in hexane were then dried under N₂. The methyl esters were redissolved in 30 ml hexane (UV grade). All the solvents used in extraction, purification and dissolution of lipids in this experiment contained the final concentration of 0.05% butylated hydroxytoluene as antioxidant. The fatty acid methyl esters were separated and quantified by gas chromatography equipped with a hydrogen flame ionization detector using a 30 m x 0.25 mm glass capillary fatty acid methyl esters column (Omegawax 250), and with on-column injection using helium as a carrier gas. A three-stage thermal gradient program was employed in the fatty acid separation, which was (a) 85°C-102°C, temperature increasing rate 30°C/min; (b) 102°C-158°C, rate 10°C/min; and (c) 158°C-245°C, rate 3°C/min. Injector and flame ionization detector temperature were 200°C and 250°C, respectively. Individual fatty acid methyl esters were identified by comparing retention times with known 16:0, 18:0, 18:1, 18:2, 18:3, 20:2, 20:3, 20:4, 20:5, 22:6 methyl esters (Sigma Chemical Co.).
III Results

III.1 *In vitro* incubations to study dose-response effects on \[^3H\]thymidine incorporation into coronary arterial explants

A. Effects of PUFAs

PUFA belonging to the \(\omega-3\) (EPA) and \(\omega-6\) (AA and ETA) families were examined at four concentrations for their effects on the proliferation of vascular smooth muscle. These data are summarized in Fig. 17.

EPA had no significant effect at 20 \(\mu M\), but vascular smooth muscle mitosis was significantly greater \((p<0.05)\) at 50, 80 and 120 \(\mu M\) than the control group value.

An ETA concentration of 20 \(\mu M\) had a significant inhibitory effect on \[^3H\]thymidine incorporation. Higher concentrations of ETA reversed this inhibition, producing a significant increase of \[^3H\]thymidine incorporation at 120 \(\mu M\). The level of stimulation of vascular smooth muscle mitosis induced by 120 \(\mu M\) ETA was similar to that of 120 \(\mu M\) EPA and 120 \(\mu M\) AA.

In contrast to both EPA and ETA, AA showed a high stimulation of vascular smooth muscle mitosis at 20 \(\mu M\). This unusual peak response with 20 \(\mu M\) AA was a highly repeatable phenomenon. This stimulation was absent at 50 \(\mu M\) of AA, but a lower level of stimulation occurred with concentrations of 80 and 120 \(\mu M\) of AA. This effect was first shown in R. Townley's BSc Honors
Fig. 17. Effects of ETA □, AA ●, and EPA ○ on [³H]thymidine incorporation into coronary artery smooth muscle from rainbow trout. Coronary artery explants were incubated with fatty acids in MHBS-1 for 48 h. Each point represents the mean±SEM of 3 determinations. The # indicates the significant differences of dpm/µg DNA value at the effects of AA from EPA and ETA at all concentrations and AA at all other concentrations (p<0.05, evaluated by Student's t-test). Asterisks indicate significant differences from the control (p<0.05).
Thesis 1992, see Appendix) and was repeated in subsequent experiments examining interactive effects of EPA, ETA and AA.

The very high stimulation of vascular smooth muscle mitosis by 20 μM AA was used to examine interactive effects of EPA and AA (Fig. 18). EPA concentrations of 20, 50 and 200 μM completely inhibited the stimulatory effect of 20 μM AA (p<0.05). In fact, the combined effects of AA and EPA were not significantly different to those with EPA alone (Fig. 17).

ETA at 2, 20 and 100 μM concentrations partially inhibited the 20 μM AA stimulation of vascular smooth muscle (Fig. 19) but was less effective than EPA in its inhibitory effects. This is interesting because ETA alone at low concentration (20 μM) could inhibit vascular smooth muscle proliferation. The combined effects of AA and EPA produced partial stimulation of vascular smooth muscle mitosis at all concentrations of ETA.

B. Effects of AA-derived eicosanoids

The effects of AA-derived eicosanoids on [3H]thymidine incorporation are shown in Figs. 20 and 21. U-46619 at 400 (1.14 μM) and 700 ng/ml (2.00 μM) concentrations significantly increased [3H]thymidine incorporation, but inhibited incorporation at 1000 ng/ml (2.85 μM) (Fig. 20). Carbacyclin had no effect at 100 (0.28 μM), 400 (1.14 μM), and 700 (2.00 μM) ng/ml, but stimulated incorporation at 1000 ng/ml (2.85 μM). LTC4 had no effect on incorporation at any concentration utilized (Fig. 20). The response to PGF2α (Fig. 21) showed a similar pattern to that of ETA (Fig. 17). Inhibition occurred at low concentration (30 ng/ml, 0.08 μM), but was reversed at higher
Fig. 18. Interactive effects of EPA and AA on \[^{3}\text{H}]\text{thymidine}\) incorporation into coronary artery smooth muscle from rainbow trout. Coronary artery explants were incubated with fatty acids in MHBS-2 for 48 h. The \(\bigcirc\) indicates the interactive effects of EPA at concentrations of 20, 50, 100 and 200 \(\mu\text{M}\) in combination with AA at 20 \(\mu\text{M}\) on \[^{3}\text{H}]\text{thymidine}\) incorporation into coronary arterial smooth muscle. The \(\square\) shows the effect of EPA alone on \[^{3}\text{H}]\text{thymidine}\) incorporation. The \(\bullet\) denotes the effect of AA at 20 \(\mu\text{M}\) on \[^{3}\text{H}]\text{thymidine}\) incorporation. Each dot represents the mean±SEM of 3 determinations. Asterisks indicate significant differences from the AA at 20 \(\mu\text{M}\) (\(p<0.05\), evaluated by Student's t-test).
EPA CONCENTRATION (pM)

- 30,000
- 20,000
- 10,000
- 0

INCORPORATION (dpm/µg DNA)

20 µM AA alone

EPA with 20 µM AA

EPA alone

110b
Fig. 19. Interactive effects of ETA and AA on [\(^3\)H]thymidine incorporation into coronary artery smooth muscle from rainbow trout. Coronary explants were incubated with fatty acids in MHBS-2 for 48. The □ indicates the interactive effects of ETA at concentrations of 2, 10, 20, and 100 µM in combination with AA at 20 µM on [\(^3\)H]thymidine incorporation into coronary arterial smooth muscle. The ○ shows the effect of ETA alone on [\(^3\)H]thymidine incorporation. The ● denotes the effect of AA at 20 µM on [\(^3\)H]thymidine incorporation. Each dot represents the mean ± SEM of 3 determinations. Asterisks indicate significant differences from the AA at 20 µM (p<0.05, evaluated by Student's t-test).
ETA CONCENTRATION (μM)

INCORPORATION (dpm/μg DNA)

- 20 μM AA alone
- ETA with 20 μM AA
- ETA alone

* indicates statistical significance.
Fig. 20. U-46619, carbacyclin and LTC₄ dose-dependent influence on [³H]thymidine incorporation into coronary artery smooth muscle from rainbow trout. Coronary artery explants were incubated with eicosanoids in MHBS-2 for 48 h. The ○, ●, and □ indicate the effects of different concentrations of U-46619, carbacyclin and LTC₄ on [³H]thymidine incorporation into coronary artery smooth muscle, respectively. Each dot represents the mean±SEM of 3 determinations. Asterisks indicate significant differences from the eicosanoids at 0 ng/ml (p<0.05, evaluated by Student's t-test).
Unit Conversion Table

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<tr>
<td>1000</td>
<td>2.85</td>
</tr>
</tbody>
</table>
Fig. 21. PGF$_{2\alpha}$ dose-dependent influences on [$^3$H]thymidine incorporation into coronary artery smooth muscle from rainbow trout. Coronary artery explants were incubated with PGF$_{2\alpha}$ in MHBS-2 for 48 h. The □ indicates the effect of different concentrations of PGF$_{2\alpha}$ on [$^3$H]thymidine incorporation into coronary artery smooth muscle. Each dot represents the mean±SEM of 3 determinations. Asterisks indicate significant differences from the PGF$_{2\alpha}$ at 0 ng/ml (p<0.05, evaluated by Student's t-test).
### Unit Conversion Table

<table>
<thead>
<tr>
<th>PGF$_{2\alpha}$</th>
<th>ng/ml</th>
<th>nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

![Graph showing incorporation (dpm/µg DNA) vs PGF$_{2\alpha}$ dose (ng/ml)]
concentrations, resulting in a significant stimulation at 120 ng/ml (0.33 μM). None of the AA-derived eicosanoids had as high [3H]thymidine incorporation as 20 μM AA.

III.2 Biochemical analysis of the PUFA composition in gill PL

Measurement of the fatty acid composition in gill PL from parr and mature coho salmon (Table 3) indicated that AA levels were the highest among the PUFAs of PI and phosphatidylserine (PS) from mature fish gills. In the mature fish the AA content in PI was significantly higher (3.7-fold) (p<0.05, n=5) than EPA. There was no statistical difference between AA and EPA content in the other three classes of PLs. In parr, AA level, were generally lower than EPA in PE, PS and PC, except in PI. The AA level in PI was higher than EPA (20:5 ω-3) or docosahexaenoic acid (22:6 ω-3).

With my analytical procedures 18:1 (ω-9), 18:2 (ω-6) and 18:3 (ω-3) were not separated with the GC. Even though these three fatty acids represent a major composition of the total fatty acids in fish tissue, the separation of these fatty acids was not considered important in the present study because these fatty acids are not direct precursors of eicosanoids.

There is now extensive evidence that the metabolism of AA in terrestrial mammals can be modulated by the presence of ω-3 PUFA, e.g., EPA, in membrane PL (Dyerberg and Jorgensen 1982). Thus, the ratio of AA/EPA is usually used as an indirect index of their potential for metabolism in mammals (Dyerberg et al. 1978, 1979; Bell and Sargent 1985). The ratio of AA/EPA in PI was significantly higher (p<0.05) in mature fish (3.7) than in
Table 3. Fatty Acid Compositions (%) of Phospholipids from Parr and Mature Coho Salmon Gills

<table>
<thead>
<tr>
<th>Fatty Acids</th>
<th>Parr</th>
<th>Adult</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>20.2±2.0</td>
<td>39.6±2.9</td>
</tr>
<tr>
<td>C18:1+2+3</td>
<td>33.2±2.8</td>
<td>4.5±1.0*</td>
</tr>
<tr>
<td>C20:2 ω6</td>
<td>5.2±0.4</td>
<td>4.8±0.5</td>
</tr>
<tr>
<td>C20:3 ω6</td>
<td>4.1±0.7</td>
<td>1.8±0.1*</td>
</tr>
<tr>
<td>C20:4 ω6</td>
<td>16.4±4.9</td>
<td>27.7±3.3*</td>
</tr>
<tr>
<td>C20:5 ω3</td>
<td>10.4±3.5</td>
<td>7.5±1.0*</td>
</tr>
<tr>
<td>C22:6 ω3</td>
<td>10.5±1.4</td>
<td>14.1±1.1*</td>
</tr>
<tr>
<td>PE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>31.7±4.0</td>
<td>13.2±0.8*</td>
</tr>
<tr>
<td>C18:1+2+3</td>
<td>13.7±0.1</td>
<td>23.6±2.7*</td>
</tr>
<tr>
<td>C20:2 ω6</td>
<td>4.7±0.9</td>
<td>3.8±0.1</td>
</tr>
<tr>
<td>C20:3 ω6</td>
<td>2.8±0.2</td>
<td>1.8±0.3*</td>
</tr>
<tr>
<td>C20:4 ω6</td>
<td>9.7±0.4</td>
<td>14.8±1.4*</td>
</tr>
<tr>
<td>C20:5 ω3</td>
<td>14.1±1.2</td>
<td>18.4±1.4*</td>
</tr>
<tr>
<td>C22:6 ω3</td>
<td>23.2±0.2</td>
<td>24.4±1.5*</td>
</tr>
<tr>
<td>PC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>27.1±1.5</td>
<td>14.3±1.1*</td>
</tr>
<tr>
<td>C18:1+2+3</td>
<td>17.5±1.2</td>
<td>11.3±1.0*</td>
</tr>
<tr>
<td>C20:2 ω6</td>
<td>6.2±0.6</td>
<td>2.2±0.1*</td>
</tr>
<tr>
<td>C20:3 ω6</td>
<td>4.3±0.7</td>
<td>1.7±0.2*</td>
</tr>
<tr>
<td>C20:4 ω6</td>
<td>11.5±0.3</td>
<td>15.8±1.1*</td>
</tr>
<tr>
<td>C20:5 ω3</td>
<td>19.5±1.7</td>
<td>18.8±2.3</td>
</tr>
<tr>
<td>C22:6 ω3</td>
<td>14.0±4.5</td>
<td>36.0±2.3*</td>
</tr>
<tr>
<td>PS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C16:0</td>
<td>18.9±1.5</td>
<td>20.7±1.2</td>
</tr>
<tr>
<td>C18:1+2+3</td>
<td>21.0±0.6</td>
<td>9.4±0.8*</td>
</tr>
<tr>
<td>C20:2 ω6</td>
<td>8.1±0.5</td>
<td>11.8±1.1</td>
</tr>
<tr>
<td>C20:3 ω6</td>
<td>8.1±1.8</td>
<td>7.2±0.5</td>
</tr>
<tr>
<td>C20:4 ω6</td>
<td>12.4±2.4</td>
<td>21.9±4.0*</td>
</tr>
<tr>
<td>C20:5 ω3</td>
<td>26.3±1.5</td>
<td>15.3±3.0*</td>
</tr>
<tr>
<td>C22:6 ω3</td>
<td>5.2±0.1</td>
<td>13.7±0.9*</td>
</tr>
</tbody>
</table>

* denotes statistical differences (p<0.05) between corresponding values for parr and adult.
Fig. 22 The ratio of AA/EPA from four phospholipid classes (PI, PS, PE and PC) in gill tissue from parr and adult coho salmon. A comparison is made between parr and mature fish. A significant difference (p<0.05, evaluated by Student's t-test) is denoted by an asterix. Each vertical bar represents mean ±SEM of results in 5 replicates.
parr (1.5) (Fig. 22). A statistically higher ratio (p<0.05) of AA/EPA in PS in mature fish (1.4) than in parr (0.5) was also found. The AA/EPA ratios in PE and PC were not significantly different between parr and mature fish and, in both cases, EPA incorporation was favoured over AA incorporation as indicated by a AA/EPA ratio of <1.0.

A higher level of AA incorporation into PI and PS of gills was unexpected given the high level of EPA known to be in the diet and muscle of mature fish. The finding is, however, consistent with the idea of preferential AA incorporation into the PI.
IV. Discussion

IV.1 Effect of PUFAs and their metabolites

My data are the first to report PUFA effects on any aspect of coronary vascular smooth muscle in fish. The exact response to PUFA depended on the specific fatty acid used and on its concentration. In general, AA, EPA and ETA had similar stimulatory effects at concentrations between 50 and 120 \( \mu M \). However, at lower, more physiological concentrations, an important difference was seen. 20 \( \mu M \) AA caused an especially marked stimulation of vascular smooth muscle proliferation, the highest seen in all experiments. By contrast, 20 \( \mu M \) ETA inhibited vascular smooth muscle mitosis and 20 \( \mu M \) EPA had no effect.

The stimulatory effect of 20 \( \mu M \) AA on fish medial vascular smooth muscle proliferation is consistent with findings for mammals. Huttner \textit{et al.} (1977) found that AA induced guinea pig aortic vascular smooth muscle proliferation at a concentration of 16 \( \mu M \) in culture. Morisaki \textit{et al.} (1983) also observed a stimulatory effect of AA in smooth muscle cells of guinea pig aorta in culture at low concentrations of 10 \( \mu M \). Thus, several investigators (Huttner \textit{et al.} 1977 1978; Kidwell \textit{et al.} 1978, and Wicha \textit{et al.} 1979) concluded that AA at concentrations around 20 \( \mu M \) may stimulate smooth muscle proliferation in mammals. Because the physiological concentration of AA in spawning coho salmon plasma (Gong and Farrell 1990) is low (0.04 \( \mu M \)), 20 \( \mu M \) concentration of AA may be a physiologically relevant mitogen for medial vascular smooth muscle proliferation in fish coronary arteries.
This being the case, further studies are warranted to quantify effects on intimal vascular smooth muscle and to establish if this sort of stimulation promotes or leads to coronary lesions in fish.

The present study also discovered important interactive effects of PUFAs. EPA and ETA at 20 μM, respectively, could completely and partially inhibit the effect of 20 μM AA on vascular smooth muscle proliferation. ETA was less effective than EPA. Again 20 μM EPA and ETA are likely physiological concentrations. In murine, EPA and ETA are reported to be effective inhibitors of cell proliferation (Morisaki et al. 1982, 1983). This effect was through EPA or ETA being competitively incorporated into cellular PL and inducing a decrease in AA content (Morita et al. 1982, 1983). The findings in the murine model may explain why 20 μM EPA alone does not show its inhibitory effect on vascular smooth muscle proliferation in fish. However, EPA did inhibit vascular smooth muscle proliferation in fish explant when simultaneously stimulated with AA. Thus, the inhibitory effect of EPA and ETA on AA stimulation of vascular smooth muscle mitotic activity could have a similar mechanistic basis as that reported for the murine model.

It is generally believed that the effects of PUFAs on coronary vascular smooth muscle proliferation in mammals are linked to their metabolites. Gerrard (1985) found that the plasma TXA₂ concentration in patients with injured vessels was increased. After vessel injury, platelets became attached to the side of the injured vessel and produced TXA₂ which stimulated further vascular smooth muscle proliferation which may induce lesion formation. Moncada and Vane (1979) and Weiner et al. (1986) also considered that increased TXA₂ was important in the generation of atherosclerosis. Akopov
et al. (1988) found that U-46619 (TXA₂ analogue) at concentrations between 50-500 ng/ml, stimulated human aortic intimal smooth muscle cells in culture.

The linkage between PUFA metabolites and vascular smooth muscle mitosis was also found in my work in salmonids. In the present study, U-46619 (TXA₂ analogue) stimulated vascular smooth muscle proliferation at concentrations between 100-700 ng/ml. Thus, the possibility now exists that the effect of TXA₂ on vascular smooth muscle proliferation may be similar in both mammals and fish.

PGF₂α is a potent mitogen (Huttner et al. 1978) and vasoconstrictor (Moncada and Vane 1979) in mammals. PGF₂α stimulated guinea pig aortic smooth muscle cell proliferation at a concentration of 100 ng/ml in culture, but was without effect at lower concentrations (Cornwell et al. 1979, Bettger and Ham 1981). Similarly, PGF₂α stimulated vascular smooth muscle mitosis in fish coronary explants at a concentration of 120 ng/ml; a modest growth-inhibitory effect was also observed at a concentration of 30 ng/ml. In addition to effects on smooth muscle proliferation, PGF₂α is also a potent vasoconstrictor of the coronary artery in the rough skate, Raja nasuta (Farrell and Davie 1991), and rainbow trout (Small and Farrell 1989). Again the possibility now exists that vasoactive and mitogenic roles of PGF₂α on fish and mammals may be similar.

Morisaki et al. (1988a) found a potent inhibitory effect of PGI₂ (7-700 ng/ml) on smooth muscle cells cultured from the thickened intima of rabbit aorta when PGI₂ was added to the culture at 12 h. Ross and Glomset (1976) also
reported that exogenously added PGI\(_2\) (2-2000 ng/ml) inhibited DNA synthesis of medial smooth muscle cells when it was introduced into culture after stimulation with serum of cells in the G\(_0\) stage. This was not the case in the present study. When carbacyclin (PGI\(_2\) analogue) was added to the culture medium at concentrations of 100-700 ng/ml, it had no effect. Moreover, carbacyclin showed a significant stimulatory effect at the highest concentration tested (1,000 ng/ml). Clearly, the effect of PGI\(_2\) on vascular smooth muscle mitosis appears different in fish and in mammals.

Leukotrienes are lipoxygenase products and AA-derived leukotrienes are important mitogens in cell proliferation in mammals. For example, LTB\(_4\), LTC\(_4\) and LTD\(_4\) were able to induce DNA synthesis in arterial smooth muscle cells in culture (Palmberg et al. 1987); to promote proliferation of glomerular epithelial cells (Band et al. 1985), and; to stimulate DNA synthesis in epidermal keratinocytes (Kragballe et al. 1985). In mammals, LTC\(_4\) has a potent mitotic activity in vascular smooth muscle. In contrast, LTC\(_4\) had no effect on vascular smooth muscle proliferation in rainbow trout. Fish tissues in general probably have an LTC\(_4\) receptor; such a receptor was found to mediate responses to LTC\(_4\) in fish gills and leukocytes (Praag et al. 1987, Bell et al. 1992). However, it is not known at this time whether or not the LTC\(_4\) receptor is present coronary vascular smooth muscle.

Although applied eicosanoids exert stimulatory and inhibitory mitotic effects on vascular smooth muscle in fish, it is important to establish that fish tissue can in fact form these eicosanoids (Henderson and Tocher 1987). Eicosanoids are known to occur naturally in fish tissue. The early work of Christ and Van Dorp (1972) showed that homogenates of gills were capable of forming
PGE$_1$ from ETA. Bell et al. (1992) and Beckman et al. (1992) reported formation of cyclooxygenase eicosanoids TXA$_2$, 6-keto-PGF$_{1\alpha}$, PGE$_2$ and PGD$_2$ from cultured gill cells in Atlantic salmon and rainbow trout. TXA$_2$ was also found to be produced from rainbow trout thrombocytes by Matsumoto et al. (1988). Besides cyclooxygenase eicosanoids, lipoxygenase eicosanoids LTB$_4$, LTC$_4$, LTD$_4$, and LTE$_4$ were found in homogenized killifish and Atlantic salmon gills cultured with AA (Praag et al. 1987, Bell et al. 1992). Bandyopadhyya et al. (1982) demonstrated that exogenous AA was metabolized to PGD$_2$, PGE$_2$ and PGF$_{2\alpha}$ by microsomes from liver, kidney and intestinal tissue from carp, tilapia and Asian catfish. Thrombocytes from carp also produced primarily PGE$_2$, PGF$_{2\alpha}$ and PGD$_2$ from exogenously added AA. In addition, EPA has been utilized as an exogenous substrate for prostaglandin production in tissue from rainbow trout. Thrombocytes and microsomes in plaice can form TXB$_3$ and PGE$_3$ (Kayama et al. 1986). So fish tissues, like mammalian tissues, can produce a variety of eicosanoids. The present studies indicate that the eicosanoids, PGF$_{2\alpha}$ and TXA$_2$, promote medial vascular smooth muscle mitotic activity of coronary arteries in rainbow trout.

IV.2 Possible mechanism of EPA and ETA inhibitory effect

There are two possible mechanisms that may explain the inhibitory effects of EPA or ETA on the stimulatory effect of AA on vascular smooth muscle proliferation found in fish.

(1) EPA or ETA added to the medium may cause a shift from PGI$_2$ and TXA$_2$ derived from AA to PGI$_3$ and TXA$_3$. Since TXA$_3$ is not a mitogen, and PGI$_3$
is similar to PGI₂ in its anti-mitotic properties, the shift from the metabolism of TXA₂ to TXA₃ metabolism by adding EPA in vascular smooth muscle may decrease smooth muscle proliferation.

(2) EPA or ETA in the medium may competitively incorporate into PL and block the oxidation of AA by fatty acid cyclooxygenase and lipoxygenase in vascular smooth muscle cells. The inhibitory effect of EPA on the oxidation of AA by cyclooxygenase in vascular smooth muscle may then result in a decrease in TXA₂ and PGF₂α formation, reducing vascular smooth muscle proliferation. These two possible mechanisms have both been supported by various experiments in mammals (Raz et al. 1977; Huttner et al. 1977; Morisaki et al. 1982; Morita et al. 1983; Terano et al. 1984; Lee et al. 1985; Strasser et al. 1985; Croft et al. 1988).

In the mammalian model, Morisaki et al. (1982) found a potent inhibitory effect of ETA on guinea pig aortic smooth muscle cell proliferation in culture. The ETA was attributed to the replacement of ETA with AA in PLs. Similarly, feeding dietary supplements of marine oils rich in ω-3 PUFA to mammals increased the content of ω-3 PUFA in cellular phospholipids (Iritani and Fujikawa 1982), with a concurrent decrease in the formation of PGE₂ from AA (Schoene et al. 1981). This result is due to a reduced availability of AA substrate from phospholipids. Dietary supplementation with EPA in both humans (Lee et al. 1985; Strasser et al. 1985) and rats (Terano et al. 1984; Croft et al. 1988) shows that EPA is a preferred substrate for both 5-lipoxygenase and cyclooxygenase, and has been shown to down-regulate the formation of LTB₄ and TXA₂. It is suggested that there is
competitive displacement of AA by EPA in PLs leading to decreased formation of AA-derived eicosanoids.

The present study also supports the mechanism that EPA and ETA may competitively displace AA in vascular smooth muscle membrane PL, reduce AA release and increase EPA production. AA alone can significantly stimulate vascular smooth muscle proliferation \textit{in vitro}; at equimolar concentrations EPA and ETA can competitively inhibit the stimulatory effect of AA.

Competitive incorporation of PUFA into PL was also reported in fish tissues \textit{in vitro}. Henderson \textit{et al.} (1985) found that EPA partially inhibited the incorporation of AA into total PL of turbot liver and intestine homogenates. Tocher and Sargent (1986) reported that at equimolar concentrations, EPA reduced [1-14C]AA incorporation by 47\% in peripheral blood neutrophils from plaice. Kanazawa's (1982) incorporation study of dietary radioactive EPA in larval fish also concluded that EPA is likely to be utilized as a constituent of cellular membranes for fish growth rather than to be oxidized for energy and metabolites.

\textbf{IV.3. The pattern of PUFA incorporation into fish gill PLs}

The present data indicate that the AA content was significantly higher than EPA in PI of gills from mature coho salmon, the ratio of AA/EPA is 3.7. This result is consistent with the results from the phospholipids of blood neutrophils from plaice (Tocher and Sargent 1986), plaice skin (Anderson \textit{et al.} 1981) and turbot gills (Henderson \textit{et al.} 1985). These authors also found
that the specificity for incorporation into PI is significantly greater with AA than with EPA. Thus, although the eicosanoid synthetic pathway is similar in both fish and mammals, i.e., via cyclooxygenase and lipoxygenase pathways (Herman et al. 1984, German et al. 1986, Praag et al. 1987, Bell et al. 1991), the incorporation ratio of AA and EPA into PI is different in a variety of fish tissues. It certainly does not reflect the abundance of ω-3 PUFA in the diet of marine fish such as plaice, turbot and maturing salmon, even though high EPA levels are reflected in other PLs such as PE and PC and in free fatty acids in general.

PS is also known as one of the sources of the precursors of eicosanoids in mammals. Its physiological role in fish is not clear. The ratio of AA/EPA was also significantly higher from mature coho salmon than from parr but to a lesser degree than PI.

Thus, even though PI is only a small component of the total ω-3 PUFA pool, it is the one critical to eicosanoid synthesis. This different incorporation ratio of AA and EPA into PI is predicted to directly affect the conversion rate of both PUFA families because the relative rates of eicosanoid synthesis from AA and EPA is likely governed by their rates of release from phospholipids (Bell et al. 1986). When relatively more EPA is incorporated into PI, EPA metabolites will be formed at a greater frequency. Conversely, if dietary EPA is not effectively incorporated into membrane PLs, it will not be converted to the EPA-derived metabolites. Instead, AA-derived eicosanoids will dominate the metabolite profile. This different PUFA incorporation rate in fish from mammals may be the key reason for coronary lesion formation in salmonids despite high levels of ω-3 PUFA in fish tissue. Since this conclusion is only
derived from the analysis of PUFA levels of PI, it needs to be further identified through a determination of eicosanoid production from competitive inhibition trials of \( \omega-3 \) and \( \omega-6 \) PUFAs in culture.

PI, PC, PE and PS are four major PLs in fish tissues. Of these, PE, PC and PS are found in greater amounts of PLs in fish tissues (Henderson and Tocher 1987). In PC and PE, EPA and other \( \omega-3 \) PUFAs were incorporated at a higher level than AA and other \( \omega-6 \) PUFAs, but there were no differences between the parr and adults. These \( \omega-3 \) PUFAs, therefore, constitute a major component of fish tissue PUFAs. They may play the role of: providing part of the cytoskeletal material to form an impermeable barrier; maintaining the fluidity of membranes, and providing a pool of fatty acids for energy. At the cellular level, various membrane interactions, such as cell proliferation, cell fusion, cell killing and many receptor functions can be influenced by membrane fluidity (Bell et al. 1986). As described in the Introduction, the type of unsaturation of fatty acids afforded by \( \omega-3 \) PUFA will tend to increase in membrane fluidity. It is not clear if PC and PE provide PUFAs for eicosanoid production in fish, but this possibility seems unlikely based on several people's work with fish (Marshall et al. 1981; Irvine 1982; Bell et al. 1986; Henderson and Tocher 1987).

In summary, gill tissue of coho salmon, and especially mature fish, preferentially incorporate AA over EPA into PI and PI is the major source for eicosanoid production in fish. Also, AA (but probably through the formation of metabolites) and AA-derived metabolites were shown to significantly stimulate medial vascular smooth muscle. However, EPA can inhibit this AA-mediated stimulation of vascular smooth muscle mitosis. If the present
studies with coronary explants can be proven to be relevant to coronary lesion formation in salmonids, it is possible to make the following speculation. The potentially beneficial effect of EPA inhibition of AA-stimulated coronary vascular smooth muscle mitosis is not expressed because of a reduced incorporation of dietary EPA into membrane PL. Furthermore, even though migratory salmonids eat and contain high levels of ω-3 PUFA, the beneficial effects of ω-3 PUFA on coronary lesion formation (as found in mammals) are also not expressed, and coronary lesion formation in migratory salmonids is extensive.

IV. 4 Conclusion

PUFA and PUFA-derived metabolites might have generally similar effects in salmonid vascular smooth muscle proliferation as in mammals. An important demonstrated exception was the effect of PGI₂. PGI₂ showed no effect on vascular smooth muscle proliferation although it has an inhibitory effect in mammals. At physiological concentrations, AA and AA-derived metabolites stimulated coronary artery vascular smooth muscle mitosis in culture. EPA and ETA inhibited the mitogenic activity of AA. The incorporation of PUFA into fish PLs does not reflect availability as it does in mammals. Salmon gill tissue apparently shows a metabolic preference of AA over EPA for incorporation into membrane PI and PS despite high levels of ω-3 PUFA in their tissue. The higher incorporation of AA than EPA into PI may release higher concentrations of AA and AA-derived eicosanoids since PI is a major PUFA supplier for eicosanoid production in fish.
Although relevant linkages between coronary lesions in salmonids and (a) medial vascular smooth muscle mitosis in coronary explants, (b) PUFA metabolites and (c) preferential incorporation of AA into membrane PI to prelude the inhibitory effects of EPA metabolites on AA-stimulated vascular smooth muscle mitosis were suggested in this thesis, my studies only provide the preliminary work for such linkages. Further work is clearly needed before a sound experimental base will exist to explain the etiology of coronary lesions in migratory salmonids despite their high dietary levels of ω-3 PUFA.
Part Three: References


Bell M. V., Simpson C. M. F. and Sargent J. R. (1983) (n-3) and (n-6) polyunsaturated fatty acids in the phosphoglycerides of salt-secreting epithelia from two marine fish species. Lipids 18:720-726.


biosynthesis either from exogenous fatty acid or release with hydralazine. Lipids 18:349-352.


Part Four: Appendixes
1. MHBBS media alterations and $[^{3}H]$thymidine incorporation

MHBS-1 with 10% FBS and 8 µCi $[^{3}H]$thymidine showed an almost two fold increase in radioisotope incorporation over MHBS-1 with 4 µCi $[^{3}H]$thymidine (Fig. A1). Finally, the medium for culturing coronary artery explants consists of MHBS-1 with 8 µCi $[^{3}H]$thymidine and 10% FBS.

2. Time-dependent growth of coronary smooth muscle

A time-dependent 2-fold increase in quantified $[^{3}H]$thymidine incorporation occurred at 24 h. However, during the 48 h incubation, a maximum 4-fold increase occurred using an EPA concentration of 40 µM (Fig. A2) compared to EPA added explant in with 24 h. This suggests that both the 24 and 48 h incubation times are long enough for effective PUFA incorporation and metabolism. However, since a higher dpm/µg DNA is preferred, the 48 h incubation time was used. Only EPA was used to obtain a satisfactory incubation time, thus, the other PUFA may need slightly different times for incorporation. However, since all the PUFA are similar in carbon length and saturation (differing by one double bond) in this study, 48 h was used as an initial incubation time for metabolic incorporation of all other PUFA.
Fig. A1

MHBBS & 4 μCi $[^3]$H]thymidine (48 h)

MHBBS & 10% FBS & 8 μCi $[^3]$H] thymidine (48 h)
Fig. A2

- **Control**
- **40 μM EPA**

- Incubation time (h)
- 

\[
\text{[^3]}H\text{thymidine (dpm/μg DNA)}
\]

- 24 hours
- 48 hours

*Significant difference