Hypoxic Conditioning in blood vessels and smooth muscle tissues: effects on function, mechanisms, and unknowns.

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Running Head: conditioning in smooth muscles

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**ABSTRACT**

Hypoxic preconditioning, the protective effect of brief, intermittent hypoxic or ischemic episodes on subsequent more severe hypoxic episodes, has been known for thirty years from studies on cardiac muscle. The concept of hypoxic preconditioning has expanded; excitingly, organs beyond the heart, including brain, liver and kidney, also benefit. Preconditioning of vascular and visceral smooth muscles has received less attention, despite their obvious importance to health. In addition, there has been no attempt to synthesize the literature in this field. Therefore, in addition to overviewing current understanding of hypoxic conditioning, we consider the role of blood vessels in conditioning and explore evidence for conditioning in other smooth muscles. Where possible we have distinguished effects on myocytes from other cell types in the visceral organs. We find evidence of a pivotal role for blood vessels in conditioning and for conditioning in other smooth muscle, including, bladder, vascular myocytes and GI tract, and a novel response in uterus of hypoxic-induced force increase, which helps maintain contractions during labour. To date however there is insufficient data to provide a comprehensive or unifying mechanism for smooth muscles or visceral organs, and the effects of conditioning on their function. This also means no firm conclusions can be drawn as to how differences between smooth muscles in metabolic and contractile activity may contribute to conditioning. We have therefore suggested what may be general mechanisms of conditioning occurring in all smooth muscles, and tabulated tissue specific mechanistic findings, and suggested ideas for further progress.

KEY WORDS: preconditioning, ischemia, contractility, smooth muscle.

**Introduction**

Hypoxia (decreased oxygen delivery) and ischemia (decreased blood flow) are relatively common stressors of cells and tissues. They cause immediate shortfalls in oxygen and nutrients. This produces alterations in the balance of several ions, including protons, inside and outside of the cell, and accumulation of other “waste” products of metabolism (31, 55, 68). Much tissue damage also occurs with reperfusion following ischemia. This is caused by the surge of oxygen producing free radicals and neutrophil infiltration, and Ca2+-loading pH regulating mechanisms being stimulated (78, 166).

As has been pointed out by many authors, the essential role of oxygen to most life forms makes it not surprising that organisms have evolved with diverse mechanisms and adaptations to cope with periods of hypoxia (35, 57, 109, 113, 125). That ischemic damage could be reduced by hypoxia, was first described for brain, in the 1960’s (40) . Then in the 1980’s Murry et al (131) found that brief and intermittent ischemic episodes could protect the heart from the consequences of subsequent longer ischemic episodes; ischemic preconditioning. Tomai et al in 1999, subsequently defined Ischemic preconditioning as “the ability of short periods of ischemia to make the myocardium more resistant to a subsequent ischemic insult” (183). Since its initial discovery around 30 years ago there has been great interest and much literature in the field of ischemic conditioning. From the initial discoveries around contractility in isolated hearts, ischemic conditioning has been shown to be protective against myocardial infarcts, myocardial stunning and arrhythmias (63, 96)

**Mechanisms of conditioning.**

*Background*

Tissues, cells and molecules all respond to hypoxia with mechanisms that are immediate if the hypoxia is acute or adaptive if the hypoxia is chronic. During hypoxia sufficient to cause an infarct or stroke, there will be cell and tissue death occurring by a variety of mechanisms. In preconditioning however, where brief (minutes) episodes of hypoxia are performed, the cellular responses that occur to these acute dips in oxygenation are different. They involve for example, metabolic responses, stabilization of hypoxia-inducible factors (HIF-1 and HIF-2), alteration of proteins e.g. phosphorylation, and changes in ionic gradients and metabolites. With preconditioning we are concerned with how these changes, which are intermittent and limited, produce the alteration of tissue response, when a longer lasting, deeper hypoxia occurs.

When considering mechanisms we may expect that the additional changes occurring with ischemia vs just hypoxia, will produce somewhat different outcomes. Hypoxic responses in tissues however lead to increased perfusion and in this way are often sufficient to also correct the ischemic changes. Thus hypoxia is a major stimulus for conditioning and it or its surrogates are used in most studies.

To tease out mechanisms, data from conditioning experiments are easier to understand when isolated preparations, limited to one or other cell type, are used. This however abolishes the natural interactions that occur in whole organs subjected to ischemia or hypoxia. This is particularly pertinent to discussion of the visceral organs, where the smooth muscle myocytes are just one of several cell types present.

*Current synthesis.*

Two of the earliest identified contributors to conditioning mechanisms were identified as hypoxia-inducible factor (HIF) and adenosine. In the 60s, Dahl and Balfour, (1964) showed that mice repeatedly exposed to anoxia developed a tolerance to it, and if an extract of their brain was given to control mice, cerebral protection was conveyed (40). The factor producing this tolerance was HIF (110). More recently it has been shown that interfering RNAs for HIF-1α expression abolishes preconditioning in the heart (54). We know that HIF plays a pivotal role in the transcriptional adaptive response to hypoxia. HIF prolyl hydroxylases sense oxygen and uses this to control HIFα degradation. Conditioning protection can be achieved using inhibitors of the propyl hydrolases (82) Erythropoietin (EPO, and its receptors) are increased by HIF and have been found to be an important part of the way it reduces ischemic injury in tissues.

Adenosine, like HIF, is integral to the mechanism of preconditioning. It is elevated during hypoxia, as ATP is hydrolyzed and subsequently deaminated, and leaves cells. Adenosine (and ATP) activates extracellular receptors, which are expressed in a cell-specific manner. Adenosine levels are higher in hearts that have been preconditioned and adenosine receptor blockers prevents remote conditioning (154). The main effect associated with receptor activation is a change in cAMP levels. This can decrease myocyte contractility and produce vasodilation, by lowering intracellular calcium (see Figure 1) and increasing the integrity of the endothelium (38, 73, 204).

Following these early discoveries many more players have been identified as contributing to conditioning (55). These include both intra- and extracellular mediators, as well as effects produced by the nervous and immune systems and changes in gene activity (102, 122). We do not present an exhaustive list but highlight mechanisms that are likely to be particularly relevant to smooth muscle, and refer readers to the several reviews in our bibliography (20, 42, 55, 65, 142, 178, 184).

Extracellular signals evoked by conditioning include, agonists, hormones and paracrine factors, cytokines and metalloproteinase inhibitors. Neuroimmune molecular responses linked to conditioning include innate immune signaling pathways such as Toll-like receptors and type 1 interferons (122). Intracellularly, many kinases and signaling factors known to be important in smooth muscles have been identified including; heme oxygenase-1, nitric oxide (NO), HIF, VEGF (vascular endothelial growth factor), ATP-sensitive K (KATP) channels, prostaglandins, bradykinin, PKC, PKG, reactive oxygen species (ROS), as well as stromal derived factor-1α and microRNA-144 (3, 25, 44, 45, 85, 132, 150, 202).

The induction of conditioning is associated with substantial changes in gene expression, both up and down regulation. In turn these cause major effects on cell signaling pathways, which will contribute to cellular protection. It has been suggested that preconditioning stimulates a “fundamental genomic reprogramming of cells that confers cytoprotection and survival” (48). As anticipated from the above discussion, changes occur in the genes and pathways involved in metabolism, immune responses, ion-channel activity, and components of the hematological system. DNA methylation and histone modification, which epigenetically modify the chromatin structure and thereby access of transcription factors to regulatory loci, are also targets in the genetic reprogramming.

Due to their intimate involvement with oxidative respiration, mitochondria have also been a focus of studies. Conditioning regimes are able to prevent the mitochondrial transition pore opening and in this way help to preserve their function, and stop the release of the caspase-activating cytochrome C (22, 71, 145). Of particular relevance to conditioning in smooth muscle will be changes in lipid rafts and caveolae and the signaling pathways associated with them. These include ion channels, hormones and tyrosine kinase associated receptors and PKC (51, 133, 188), as well as extracellular matrix and cytoskeletal proteins (51). Xu et al. (196) have recently reviewed how caveolae and their adaptor proteins, cavins may be potential therapeutic targets for ischemic disease and conditioning. Caveolin knockout mice display increased ischemic injury, whereas caveolin provided protection against ischemia and reperfusion injury. Endothelial nitric oxide synthase (eNOS) which is targeted to caveolae in blood vessels may be an important effector of the conditioning (56, 201).

Figure 1 shows how these mechanisms of conditioning can impact on signaling pathways and functioning, in a generic smooth muscle myocyte.

**Types of ischemic conditioning**

*Introduction*

Ischemic preconditioning is a highly conserved mechanism now known to be present in a variety of organisms and tissues. Ischemia or hypoxia are not the only stimuli to conditioning effects. Other stressors including, heat shock, lipopolysaccharide, pacing and exercise, can also elicit organ protection, termed cross tolerance or cross conditioning (9, 111, 116, 153, 161, 199). Our focus however is on hypoxia and ischemia. The different types of conditioning are summarized below.

*Classical and Delayed Preconditioning.* Ischemic preconditioning as described above, is also referred to as “classical” or “early” preconditioning. Investigators distinguish between mechanisms that arise in the minutes following preconditioning, (immediate), and have protective effects that last for an hour or two, and those effects and mechanisms occurring within 12-24 hours and lasting 2 to 3 days. (98, 115, 176). The later phase of protection is known as delayed, or late or SWOP (second window of preconditioning, see (76). In their review of SWOP the latter authors describe the therapeutic effects as, robust and ubiquitous but not as powerful as early Ischemic preconditioning. When considering mechanisms it is obvious that ischemic effects on gene expression and protein synthesis can contribute to SWOP but not to immediate Ischemic preconditioning, and that acute metabolic and signaling pathways that arise as an immediate consequence of ischemia, may have passed or been mitigated by the time of SWOP.

*Remote conditioning.* Remote conditioning is when periods of ischemic preconditioning protocols in one site can protect a distant organ from subsequent ischemia. Remote conditioning (several bouts of ischemia followed by reperfusion) is now understood to work at the level of vascular beds as well as tissues and organs. This effect was initially demonstrated by repetitively occluding the mesen­teric, femoral or renal arteries and seeing beneficial effects on the heart (19, 64, 180). In 2002 a blood pressure cuff on the contralateral arm was used in humans and shown to help preserve endothelial function during subsequent ischemia–reperfusion of the other arm (95).

As noted by Heusch et al (2015) for the heart, the peripheral stimulus can be chemical, mechanical or electrical and involve activation of peripheral sensory nerves (80). Serum or solutions taken from hearts or cells that have undergone Ischemic preconditioning protocols can be used to protect a distant organ from ischemia (86). Studies have also shown that remote conditioning increases NO. This may be because of shear-stress-related stimulation of endothelial NOS, secondary to reactive hyperemia at the remote site. As was found for classical conditioning, remote conditioning can be blocked if NO production is inhibited or ablated, pharmacologically or genetically, (21, 151).

Remote ischemic preconditioning has been shown to be effective in mitigating ischemia-reperfusion damage in heart, liver, kidney, brain and intestine (103). It has the potential to transform the impact of ischemic preconditioning, by performing preconditioning in accessible tissues e.g. a limb, to benefit anticipated decreases in perfusion ahead of surgery and transplants in less accessible organs, (103). In 2010 one of the first clinical trials of remote conditioning reported. Myocardial infarction patients given conditioning in the ambulance ahead of percutaneous coronary intervention, had improved myocardial outcomes (24). More recent results have however found no effects (124).

*Post-conditioning*. This refers to repeated non-lethal bouts of ischemia and reperfusion being applied for therapy shortly *after* severe ischemia, i.e. blood flow is interrupted in the reperfusion phase. Repeated brief coronary occlusion during early reperfusion reduces infarct size (70, 203). Post-conditioning may be remote or local. The attraction of post-conditioning is that the reperfusion stage is usually much easier to predict than the ischemic attacks. (107)

The mechanisms suggested for post-conditioning are in part, specific to it and reflect the fact that much tissue damage occurs as a consequence of reperfusion itself (62). Thus interruption to the surge of oxygen, by post-conditioning is thought to reduce the production of free radicals and neutrophil infiltration. It will also reduce the Ca2+-loading pH regulation mechanisms stimulated by the acidification that occurs during ischemia. Other mechanisms may be common to classical ischemic preconditioning and include bradykinin, adenosine and protein kinase signaling cascades (30, 94). In a review of post-conditioning and liver ischemic-reperfusion injury, Theodoraki et al (2016) suggested that other protective mechanisms were due to upregulation of NO and thus better flow through the hepatic circulation and improved preservation of mitochondrial structure and function (181). In heart the preservation of mitochondria may be due to post-conditioning inhibiting opening of the mitochondrial transition pore (79). In the kidney the benefits of post-conditioning for function found in animal studies, have yet to be seen in human clinical trials (89). The neonatal brain of infants suffering from hypoxic insult before or during labour and delivery, presents a highly worthwhile focus for therapy based *after* the causative event. Post-remote conditioning by mechanisms, such as limb ischemia has been suggested as a novel way forward (74).

*Natural Preconditioning.* As the clinical interest in conditioning has grown, it has also been appreciated that brief periods of conditioning hypoxia and ischemia may occur naturally and be protective. Perhaps the most readily understood form of natural preconditioning is angina. Angina, due to brief ischemia from coronary vessel spasm or block, may be considered a form of conditioning. Pre-infarction angina has been shown to be associated with reduced infarcts (4, 138, 156). Another probably related natural conditioning is that called “warm-up” or “exercise-induced angina”, whereby exercising to the point of cardiac ischemia reduces subsequent exercise-induced ischemia (99, 189). Transient ischemic attacks (TIA) are short episodes of local reductions in blood flow causing self-limiting neurologic dysfunction and no enduring injury. Data from two retrospective studies looking at the severity of stroke outcomes, supported a role for natural conditioning via TIA (128, 187), and some (165, 171), but not all (87), subsequent studies have added to the evidence suggesting that there is endogenous preconditioning in the human brain. The uterus is another organ that may be considered to exhibit natural preconditioning, as its frequent contractions produce ischemia, and aberrations in activity are associated with difficult labors (152). The details of this and the related phenomenon of hypoxia-induced force increase in the myometrium are described in the smooth muscle section later.

*Pharmacological conditioning:* This refers to the use of agents and drugs e.g. adenosine, erythropoietin, volatile anesthetics and inhibitors of HIF activation, to induce ischemic tolerance. The choice of these agents arose as details of the mechanism of conditioning emerged. The use of such agents is attractive as they are viewed as less risky than performing cannulation, surgery or producing ischemic events in organs.

**Conditioning in smooth muscle organs**

*Conditioning beyond the heart*

Unsurprisingly given its therapeutic potential and intriguing biology, investigators in other fields turned their attention to ischemic preconditioning. The brain and organs for transplant, especially kidney and liver, have received considerable attention, (1, 5, 97, 159, 197). In these organs it is clear that preservation of function is required, not only for the organ-specific cells and structures, but also their vasculature. Thus questions arose as to whether vascular myocytes contain conditioning mechanisms similar to those found in cardiac myocytes, and whether endothelial function preservation is part of the therapeutic protection. Work has progressed to study preconditioning in smooth muscles.

*Smooth muscle activity and metabolism may influence conditioning*

It is worth considering whether conditioning should be expected in smooth muscle cells. This is because aspects of their metabolism, response to hypoxia and contractility patterns are markedly different from cardiac muscle. Even between smooth muscles there are large differences. For example, the action potentials, large inward Ca2+ current and strong phasic contractions of the myometrium (88, 170) make it closer to cardiac muscle than a slow, inexcitable, tonically active blood vessel. Peristaltic contractions, i.e. waves of contraction and relaxation, such as occur in the gastrointestinal tract and ureters, are unique to smooth muscle. Skeletal muscle can enormously increase its oxygen consumption e.g. 100-fold with activity (148), whereas no smooth muscle sees such marked changes in oxygen consumption. When directly comparing oxidative capacity in cardiac, skeletal and vascular smooth muscle, that of vascular was significantly lower than the other two (140).

Typically the functions of neuronal and cardiac tissues, both metabolically highly active, are known to be very sensitive to oxygen delivery. These are also the two tissues where conditioning has been most convincingly demonstrated. For both brain and heart, hypoxia rapidly causes damage and tissue death, whereas smooth muscles are more resistant to its effects. The question therefore arises whether the different smooth muscles, with their differing activity, i.e. tonic and phasic contractility, and metabolic demands, would lead to different sensitivities to hypoxia and preconditioning. Thus when reviewing the effects of hypoxia and evidence for conditioning, it would be useful to know if the effects described are mostly due to myocytes or the other cell types in the tissue, e.g. neuronal., epithelial, endothelial.

There is another reason for asking if conditioning will be similar in smooth muscles to cardiac muscle. Starting with the work of Edith Bulbring’s group on gut, in the 1950’s, and Ric Paul’s group in the 1980’s, it became clear that there is a high degree of metabolic compartmentalizing in smooth muscles. Oxidative phosphorylation directly supports contractile activity, while ionic regulation, especially Na-K ATPase, is supported by the ATP generated from anaerobic metabolism (112, 144). There is direct interaction between these glycolytic enzymes and subunits of V-type ATPases, including the Na-K ATPase and the H-ATPase. Smooth muscles, including tonic ones, such as carotid artery and phasic, such as myometrium, are producing considerable amounts of lactate even when oxygen supplies are not limiting (112, 191). The compartmentalization of metabolism in smooth muscle was further supported by the demonstration that the enzymes for glycolysis are located at the plasma membrane, a conclusion in keeping with finding in erythrocytes (121). It is now accepted that glycolytic enzymes are membrane bound, and anchored by F-actin (46). Electrical activity and oxygen consumption are related to smooth muscle contraction but are dissociated if oxidative metabolism is inhibited; electrical activity is maintained but not the force (28).

While no smooth muscles are efficient compared to striated muscles, they do contract economically (77, 143). Smooth muscle contraction and relaxation are slower than in striated muscles. This is partly because it relies on Ca2+-regulated phosphorylation of myosin rather than the Ca2+ troponin system, and partly due to the slower release of inorganic phosphate, resulting in a slow rate of crossbridge cycling. As with striated muscles, good control of Ca2+ and pH are important to smooth muscle function and both make energetic demands on the tissue, and thus require ATP (8, 58, 192). Due to the slow release of inorganic phosphate from the cross bridge there is a relatively longer lasting attachment period and force production, per ATP hydrolyzed in smooth muscles. The oxygen consumption of smooth muscle is low compared to other muscle types, and thus their sensitivity to hypoxic stress and damage might be expected to be reduced. Compared to striated muscles however, smooth muscles have very low stores of phosphocreatine (43, 144, 191). Despite this, due to their lower energy consumption, metabolism can keep up with contraction during normal smooth muscle activity and no large increases in oxygen consumption occur (143).

Having thus set the context, we now consider what the literature reports for conditioning in vessels and visceral organs.

**Blood vessels**

With very few exceptions (avascular tissue) cells and tissues are dependent on being perfused by the circulation and are affected by ischemia (173). All orms of conditioning require reperfusion after the circulatory interruption, be that an infarct or transplantation. For this reason, conditioning needs to protect the cells of the circulation and thus a focus on blood vessels is important. Blood vessels are composed of several interacting cell types, i.e. endothelial cells, pericytes and myocytes (see (23)). In additionthe red blood cells, also shape vasculature oxygen transport (12)

Mechanisms of conditioning that affect endothelial cells, are important to the functioning of smooth muscle. Furthermore protective mediators released by conditioning endothelial cells, such as NO, will diffuse to the myocytes where they are expected to bring about conditioning (141). Endothelial cells also play a role in activating pericytes e.g. by secreting VEGF and TGF-β (158). Although pericytes react to hypoxia and may play a role in angiogenesis, we can find no literature on their role in conditioning (118, 195).

Most work exploring endothelial cell dysfunction has been carried out on coronary blood vessels but we anticipate that the findings will transfer to other vessels (149). With its key role in sensing and responding to flow and pressure changes, its anti-thrombotic role and influence on tone via signaling to myocytes, any damage to endothelium has the potential to rapidly become serious. Myocytes which exist throughout the vascular tree, apart from capillaries, will also be affected by ischemia, and subject to a similar cascade of responses and functional detriment, as has been well described for cardiac myocytes (135).

The net effect of the ischemia changes in the blood vessels will manifest as an increase in vascular permeability and hence edema, impaired vasomotion, intravascular cell aggregates forming, and even, capillary destruction with hemorrhage (91). The damage to blood vessels, and hence the tissues they supply, arises both from the ischemic period and the reperfusion. Indeed the latter is considered to be more serious, as inherent mechanisms to cope with hypoxia are suddenly confronted with restored oxygen delivery. Overload of both Ca2+ and perturbation of pH regulating mechanisms rapidly ensue and are augmented by an inflammatory response, causing cell damage and death (55). Ischemia-reperfusion can profoundly affect endothelial cell function, and a key disturbance is the decreased production of NO and increase in reactive oxygen species (ROS). As well as the predicable increase in blood pressure, there will also be deleterious effects on platelet function and inflammatory reactions (53).

Impaired vasomotion is another hallmark of vascular damage. Vasomotion produced by myocytes, is the outcome of integration of signals from all three cell types, i.e. pericytes, endothelial cells and myocytes, and contributes much to the health of the microcirculation, facilitating perfusion and responses to vasomodulators (23). Although vasodilation will be the response of most vascular beds to hypoxia and ischemia, ultimately, vasodilation is impaired, as the insult persists. In contrast, it seems that vasoconstriction of the vascular myocytes remains largely intact. Thus when constrictor mediators, (serotonin, endothelin, thromboxane) are released due to cell damage and infiltration, their effects may be greater than in a healthy vessel (11).

Specific studies in vessels have shown that intermittent hypoxia conditioning is an effective way to improve NO bioavailability and improve functional outcomes and prevent hypertension (114). Clinical studies have also found long-lasting antihypertensive effect of preconditioning blood vessels. In vascular myocytes KATP channels have been identified as a possible target of conditioning regimes (160). Via the mechanisms discussed earlier, these changes condition the endothelial and muscle cells, so that when deeper hypoxia or ischemia occurs, they are protected. In this way, better flow and pressure are maintained to tissues and organs, less damage occurs and recovery is enhanced. While we conclude that conditioning occurs in myocytes and endothelial cells, it is surprising how few studies have directly studied its effects on vascular myocytes.

**Other smooth muscles**

Searching the literature for terms involving conditioning and smooth muscles reveals a small body of work for conditioning in smooth muscles. We have, however, found studies in stomach, intestines, urinary bladder and uterus. The specific mechanisms involving smooth muscle have been tabulated in Table 1.

**Gastro intestinal tract**

The cells that make up the gut, including smooth muscle myocytes, are susceptible to episodes of ischemia from a variety of causes, including blockage or damage to blood vessels, strangulation and internal blockages. The small intestine in particular is considered a tissue that is very sensitive to ischemic reperfusion damage (174). Intestinal ischemia is common and has high morbidity and mortality rates. Damage to mucosal or epithelial cells will affect gut function and smooth muscle contractility, as will infiltration of cells from the immune system, chemokines, and direct effects of the hypoxia on contractility. Furthermore GI motor function is dependent upon enteric nerves which are also susceptible to ischemic reperfusion injury. In fact this damage has been suggested to be the main cause of contractility dysfunction (13). Reduced contractility and agonist sensitivity led to delayed transit times after ischemic reperfusion injury (75). Dysfunction of intestinal smooth muscle is one of the causes of transplantation rejection (164). Gut mucosal injury triggers a systemic inflammatory response which can contribute to multiple organ failure. Investigators have therefore studied if conditioning can ameliorate some of the deleterious effects of hypoxic reperfusion on the gastrointestinal tract. Several reports have shown that preconditioning is effective in attenuating ischemic damage in the gut, specifically in stomach and intestines,.

*Intestines*

Starting from Tsuruma , et al. (185) there is now ample evidence that conditioning provides protection to the small intestine. An accumulation of fructose 1,6, biphosphate after conditioning was reported to contribute to the protection observed in intestinal preconditioning (174). In an *in vivo* study in rats, hyperpermeability of the intestinal epithelium and reduction in villus height was attenuated by transient occlusions of the mesenteric blood vessels (120). The preconditioning effects were shown to be, at least in part, mediated through adenosine, NO, PKC and haemoxidase. In more recent studies, conditioning was found to upregulate the expression of intestinal stem cell markers, (33). Also increased was HIF-1a and Wnt-/b-catenin expression in the intestinal crypts (34). Pinheiro et al found that conditioning protects the small intestine from ischemic injury by increasing systemic inflammatory mediators (CINC-1 and CINC-2) (146). Further insight into the mechanism of conditioning in the intestine was provided by a study of Toll-like receptor 4 (TLR4). This is considered a key receptor in transduction of intestinal inflammatory responses. Small intestinal ischemia-reperfusion injury in rats was ameliorated by the use of post-conditioning, attributed to the decreased mucosal expression of TLR4 (162). Suppressing of cytokine signaling-1 provides protection by degrading tumor necrosis factor receptor-associated factor 6 (TRAF6), a key adaptor protein downstream of TLR4, (105, 162). Late conditioning of intestinal tissue also is effective in improving intestinal function. It decreases inflammation and provides mucosal protection in the intestine (130). Of note, there was improvement in transit time due to better contractility. Remote ischemic preconditioning also protects the bowel (and pancreas and liver), by modulating inflammatory mechanisms (29).

Improvements in contractility in responses to electrical field stimulation and high-K depolarization, were found in studies of rat jejunum after conditioning, along with histological improvements in the enteric nerves (177).

Conditioning has been shown to be potentially useful for clinical applications such as intestinal transplants and recovery from ischemia, as well as preventing leaky gut associated with ischemia causing sepsis and shock. Investigators have therefore sought to find pharmacological surrogates to induce conditioning. Many experimental approaches have used clamping of the mesenteric vessels. Preconditioning with erythropoietin has been shown to reduce ischemic reperfusion injury in several studies of intestinal tissue. Sayan and colleagues demonstrated that it also improved physiological function (163). Ileal longitudinal muscle contractility was improved in a dose-dependent manner and attributed to the antioxidant effects of erythropoietin and effects on K channels. Other chemical conditioners effective in the gut include: the opioid receptor agonist remifentanil, (36, 169), which is already in use clinically to pre-and post-condition the heart, e.g.(190) via activation of PI3K/Akt and ERK pathways (50); and the volatile anesthetic agent, sevoflurane, which ameliorated intestinal damage, pre or post injury, by reducing apoptosis by activation of PKC and the mitochondrial KATP channel(104). In the small bowel a protective effect of conditioning treatment with hyperbaric oxygen in reducing ischemic damage was shown(18) when it was administered early in the ischemic period.

*Stomach*

Preconditioning was found to attenuate the mucosal damage in the stomach caused by ischemic reperfusion (139). The gastric preconditioning mechanism was shown to involve prostaglandin, adenosine (via A1 receptors) and nitric oxide. Specifically, if these pathways were inhibited ahead of the gastric preconditioning, the protective effect on mucosa was abolished. Classical and remote preconditioning were reported in rat stomach with the gastroprotection105 and hyperemia being attributed to both vagal and sensory nerves. Their release of vasodilatory mediators such as CGRP (27), led the authors to suggest that the gut-brain axis is important for gastric conditioning. Interestingly, culture medium from hypoxia conditioned endothelial cells protected human intestinal cells from hypoxia/reoxygenation injury (86)

In summary, there is good evidence that conditioning, pre-, post and remote is effective in the gastrointestinal tract to mitigate the effects of ischemic reperfusion injury via a variety of mechanisms, and translation to the clinical setting is occurring. The focus of these studies has been on the mucosa and epithelial cells, as well the smooth muscle cells. Improvements of transit time, morphology and contractility when directly measured, can be taken as supporting evidence for conditioning occurring in smooth muscle of the gut, with a variety of putative mechanisms being suggested, from free radical scavenging, prevention of migration of inflammatory cells to K channels. The bulk of evidence however suggests that it is other cell types in the gut that are more prone to hypoxic and ischemic damage, and that these cells also benefit from conditioning.

**Urinary System**

Ischemic kidney damage arises from a variety of causes, including shock and vascular problems, and in preservation and transfer for transplantation. Conditioning has been found to increase the resistance of kidney to ischemic reperfusion injury via a variety of mechanisms (92). Ischemic preconditioning also restricts fibrosis which develops as a result of the reperfusion injury post ischemia (182), and decreases the expression of alpha smooth muscle actin (α-SMA). Pharmacologic agents activating the HIF pathway, improve renal function in ischemic acute renal failure (17). These effects are likely to be due in part to improved vascular function as well as effects on the nephrons. Indeed using a genetic approach, the endothelial cells of renal blood vessels have been shown to be the predominant cell type in mediating the protective effects of conditioning in kidney, via modulation of endothelial vascular cell adhesion molecule 1 (VCAM1) expression and inflammatory cell adhesion (93). As with the GI tract, much interest in conditioning in the kidney has been spurred by the hope of improving the function of transplanted organs.

Focusing on smooth muscle there is evidence for conditioning in bladder. Bladder dysfunction related to ischemia arises if bladder is over-distended as can happen with urine retention and outlet obstructions (69). Damage during ischemia and reperfusion arises to nerves and smooth muscle from similar mechanisms to those identified in other tissues, such as reactive oxygen species, depleted ATP and damaged mitochondria (175). More specifically there is evidence for: impairment of myofilament proteins (84), including their packing and an irregular distribution of sarcoplasmic dense bodies (equivalent to Z-bands) in the damaged smooth muscle cells (67) and impairment of Ca2+ pump activity (179).

Lorenzi et al (108) appear to be the first to have looked for evidence of conditioning in urinary bladder. Using guinea-pig detrusor smooth muscle strips they measured force produced in response to electrical field stimulation or carbachol, under control and then zero oxygen and glucose to mimic ischemic conditions. These experimenters were able to show that the fall of force with ischemia was significantly reduced if the strips had been preconditioned by ischemia. In particular the responses to carbachol were so good after preconditioning that no significant difference from control was found. As was the case for the enteric nerves in the GI tract, this implies that the nerves within the bladder are sensitive to ischemic damage and their conditioning contributes to recovery of smooth muscle function. Yu et al also demonstrated hypoxic preconditioning protecting the urinary bladder’s function in rat. They showed that the preconditioning up-regulated expression of the anti-apoptotic protein Bcl-2, which decreases ROS damage and thus bladder injury (198). Then in 2007, Hisadome et al (2007) performed *in vivo* studies in rats. Ischemia and preconditioning were achieved via clamping the abdominal aorta and cystometric and contractile measurements made (83). The preconditioning was found to reduce bladder dysfunction due to ischemia. Infiltration of leukocytes and rupture of the microcirculation were still found after preconditioning but without sloughing of mucosal cells.

More recently, pharmacological preconditioning has been examined. Ohmasa et al focused on ATP-sensitive K+ channels in an *in vivo* model of urinary retention and *ex vivo* determination of contractility (136). Previous work from this team had demonstrated that free radicals and neutrophils contributed to bladder smooth muscle damage with urinary retention and that KATP channel play a role in this dysfunction (137). They then went on to show the damage could be reduced by using KATP channel openers, and this led the way to them testing these drugs as preconditioning agents. Specifically, nicorandil and cromakalim prevented bladder dysfunction via activation of KATP channels with an inhibition of oxidative stress and a decreased apoptosis index. Furthermore, the protective effect of KATP channel openers on bladder contractility was shown directly (136).

Proper functioning of the smooth muscle in the ureters is critical for transporting urine from kidney to bladder (59) and is dependent on a variety of Ca2+ signaling mechanisms (52, 167). Infection, as commonly occurs with *E.coli* and obstruction, are causes of ureteric smooth muscle dysfunction (47, 60). While ischemia and hypoxia are considered to act via similar pathways associated with dysfunction in other smooth muscles, we can find no studies investigating how conditioning may help preserve ureteric function, and thereby presumably contribute to recovery from disease and renal transplant. There is also no literature concerning the urethra. We conclude that endothelial cells are a prime target to protect the kidneys via conditioning. In the bladder there is good evidence for myocytes, as well as nerves, producing protection from ischemic damage.

**Reproductive tract**

The female sex steroid hormones are protect against coronary heart disease as well affecting the contractility and receptiveness of the uterus (194). Work has also shown that estrogen pre-treatment can protect against ischemic damage and suggests it plays a part in conditioning (10, 90, 147). Studies on conditioning in the reproductive tract are sparse but interesting.

Of Interest is the hypothesis that menstruation is a facilitating preconditioning for successful pregnancy, as both are associated with oxidative stress and ischemia-reperfusion injury (26). The authors note that, “reactive oxygen species production, activation of redox-sensitive signaling pathways, expression of angiogenic factors such as vascular endothelial cell growth factor, and resistance to cell death are major effectors in the process of preconditioning, all of which are highly regulated in the endometrium in response to hormonal signaling”. There is reason to predict that natural conditioning could occur in the endometrium, as blood flow changes throughout the menstrual cycle. There is relative hypoperfusion in the early and middle secretory phase and hyperperfusion in the late secretory phase. Vascular endothelial growth factor is regulated by hypoxia, and is thought to be part of the mechanism of conditioning; its expression in the endometrium changes during the menstrual cycle (168). Interestingly, VEGF mRNA expression in endometriosis patients was significantly higher than in healthy women during the late secretory phase and menstruation (49). This led Ren and colleagues to hypothesize that ischemia in the endometrium in early and middle secretory phase and reperfusion in the late secretory phase might play an important role in endometriosis by up-regulating VEGF expression (155). They found that primary human endometrial cells and endometrial tissue, when exposed to hypoxia preconditioning and then transplanted onto the chick embryo chorioallantoic membrane, showed regulated vascular endothelial growth factor expression and decreased apoptosis of endometrial cells. The authors suggest that this may be a mechanism facilitating endometrial fragments’ ectopic implantation.

Focusing on the smooth muscle of the uterus, the myometrium, it is well known that it contracts strongly in labour. The repetitive and intense contractions of labour are required to dilate the cervix, so that the fetal head and then body can pass through the birth canal, being propelled by waves of contraction of increased amplitude and frequency (193). In all species studied, these contractions compress the myometrial blood vessels (100) and are a normal part of labour. The deleterious effects of prolonged hypoxia due to severe occlusion of uterine vessels, has been well documented in animal and human uterine tissue (72, 127, 129, 157). There is also interest in whether post-conditioning could be used to mitigate some of the neuronal damage that follows ischemia insult at or around the time of birth (74).

Recently, a response similar to preconditioning has been demonstrated in the myometrium. We have found that applying frequent brief hypoxic periods using nitrogen to reduce pO2 to around 2-4%, produced successive and sustained force increases on return to normoxia (3). These increases in contractile strength were found in rat and human myometrium, but only occur in near-labour or laboring uterus. This novel finding has been called HIFI: Hypoxia-induced force increase, (3). Adenosine, ATP and COX were found to play major role in initiating the HIFI effect. This conditioning presumably contributes the contractile drive needed for parturition, where hypoxia is also transiently experienced, as the strength of contractions leads to occlusion of uterine blood vessels. It is worth noting that the HIFI effects were directly due to smooth muscle, not other cell types.

The uterus appears to be the only reproductive smooth muscle in which a functional analysis of conditioning and its effect on ischemic or hypoxic damage has been studied. Conditioning has been found in testes (32, 200) and ovaries (6, 41) but does not involve smooth muscle. In corpus cavernosa increased smooth muscle markers and increased intracavernosal pressure were found with hypoxic conditioning in repair of diabetic erectile dysfunction in rats. This however was achieved by conditioning adipose-derived mesenchymal stem cells (186), and not directly the myocytes.

**Respiratory System**

Ischemia-reperfusion damage plays an important part in several lung pathologies, such as, thromboembolism, trauma, thermal injury, and can affect the success of lung transplantation. Thus, there is a keen interest in whether conditioning can be used to improve clinical outcome. In addition infection and pulmonary inflammation are associated with acute lung injuries, with markedly increased endothelial cell permeability (117). Hypoxic preconditioning increased survival in cultured pulmonary artery endothelial cells subjected to lipopolysaccharide-induced infection, and was associated with decreased Toll-like receptor 4 expression (2).

There is also applicability to high altitude physiology and the problems of mountain sickness discomfort through to cerebral and pulmonary edema, due to severe vasoconstriction. Berger et al (16) drew parallels between the mechanisms of conditioning e.g. stimulation of nitric oxide synthase, increase in antioxidant enzymes, and downregulation of proinflammatory cytokines, to the pathways thought to be involved in high-altitude pulmonary edema, namely decreased bioavailability of NO and increased generation of ROS. They therefore investigated whether conditioning could ameliorate some of these effects. Their results have been mixed, with early reports suggesting that remote preconditioning can delay onset of acute mountain sickness (14, 16) but a more recent follow up study finding it does not prevent mountain sickness (15). In contrast Foster et al (61) found prophylactic ischemic preconditioning was associated with improved oxygen saturation and attenuation of the normal hypoxic increase in pulmonary artery pressures following ascent to high altitude.

In common with the visceral smooth muscle tissues, the effects of conditioning are likely to be on several cell types when lung preparations are used. Transient occlusions of the trachea have been performed as a putative mechanism for strengthening the diaphragmatic muscle, to help improve outcomes from ventilator weaning; ventilation being known to cause stress and atrophy of the diaphragm (172). The diaphragm is of course a striated muscle and we can find no study assessing changes in the tracheal smooth muscle under these conditions. As mentioned above for corpus cavernosa, hypoxic preconditioning of stem cells can enhance beneficial effects. Lungs subjected to hypoxia-reperfusion had less injury when preconditioned mesenchymal stem cells were given (39, 106). The former authors noted that hypoxia, “induces the expression of prosurvival mediators, chemoattractants, and growth factors involved in cell proliferation, migration, angiogenesis, antioxidant, antiapoptotic, and antifibrotic properties in mesenchymal stromal cells, optimizing their lung repair capability in an animal model of idiopathic pulmonary fibrosis”. Liu et al noted that the lung injury was reduced through anti-oxidant, anti-inflammatory and anti-apoptotic mechanisms, with endothelial cells being a key target (106).

In a study of canine tracheal smooth muscle, *in vitro* preconditioning effects on the muscle’s contractile responsiveness was investigated (126). The study however was limited to parameters associated with establishing control conditions for such studies, not on transient repetitive periods of hypoxia or ischemia. Similarly another study investigating hydrogen preconditioning of *ex-vivo* lungs ahead of transplantation (134), was limited to ventilating with or without 2% hydrogen. The authors noted an improvement in the function, but preconditioning protocols involving transient repetitive episodes of hydrogen inhalation, were not studied. It appears that the smooth muscle of trachea and bronchi have not been directly examined for the effects of conditioning on their contractility and signaling.

**Clinical Impact of Conditioning**

Implementing and translating conditioning regimes into the clinical arena is challenging. Most of the clinical trials performed have been limited to the heart, and there has been disparities in the results from laboratory studies. Animals used in research have no comorbidities or multiple risk factors, are relatively young, live in an unchanging environment, and are genetically identical. Most of these are factors difficult to bring to the study of clinical medicine, which can lead to the disparities (119). Gender will also make an impact as women, for example, are more prone to coronary vasospasm and, endothelial dysfunction than cardiac arrests (66, 101). Clinical trials of conditioning have taken place for cardiac, cerebral, hepatic and renal tissues with mixed results (37, 81). For smooth muscles we are not aware of a focus on considering how well vascular smooth muscle function, or other smooth muscle tissue, have performed in clinical trials.

**Conclusions**

Having reviewed the literature concerning smooth muscle and conditioning, there is evidence for hypoxic conditioning in several smooth muscles. We would stratify the strength of evidence in the different smooth muscles as: GI tract> uterus> urinary >vascular >respiratory. It is clear however that apart from vascular, the field is not well developed. Even when studies have been made, smooth muscle function has not always been an outcome and conclusions are based on histology or biomarkers. In some smooth muscles, for example detrusor, the beneficial effects are perhaps mostly due to better nerve survival, leading to improved function, rather than the myocytes *per se*. This is similar to blood vessels where, as well the contribution from myocytes, beneficial effects of conditioning on endothelial cells underpin a large part of the recovery of perfusion. These extra-myocyte factors are shown in Figure 2. We conclude that in several visceral organs, other cell types, be they epithelial, endothelial or neuronal, are more susceptible to hypoxic damage than myocytes, and are therefore more likely to benefit from conditioning protocols. Many smooth muscles have myocytes that contract slowly and economically, reducing their relative sensitivity to hypoxia and ischemia. The finding of hypoxia-induced force increase in myometrium is a novel form of conditioning which may be part of the process of contractility increase required for labour. It is of interest to note that the myometrium at this time is a powerful muscle, possessing large phasically active cells, which generate substantial inwards currents and are not dependent on nerves for their pacemaking. In this sense they may be viewed as more similar to cardiac muscle than many vascular smooth muscles.

More basic science studies of smooth muscle tissues to provide data and a comprehensive picture or unifying mechanism for smooth muscles and the effects of conditioning are required. These data will help us to understand whether differences between smooth muscles occur because of their different patterns of activity and metabolic needs. This will also inform us as to whether the targets of conditioning are likely to be myocytes, or rather the other cell types in the visceral organs.

We suggest as a starting point relevant expert groups might perform conditioning protocols on isolated smooth muscle preparations, especially, ureter, urethra, trachea, bronchial smooth muscle and blood vessels. Both stimulation protocols and drugs can be used to explore the role of nerves in the responses, and histology used to track cellular damage and preservation. These investigations should then determine which of our general mechanisms identified in Figure 1 apply, and which may be tissue specific and added to our Table 1. Only when we have these data can the benefits of conditioning to smooth muscle tissues for the benefit of health be appreciated.

**Figure Legends**

**Figure 1: Mechanisms of conditioning in smooth muscle cells.**

Hypoxia causes the release or production of agonists such as bradykinin and adenosine. Their binding to receptors activates G*α*q/11 which activates PLC-β, which in turn hydrolyses PIP2 to IP3 and DAG. The DAG activates PKC. Adenosine will also increase NO and COX 1 and 2, leading to prostaglandin production (not shown on figure).Bradykinin is also suggested to cause the opening of voltage-operated Ca2+ channels and thereby promote Ca2+ entry and force. Inhibition of the Ca-ATPase pump by hypoxia inhibits Ca2+ exit from the cell, thus promoting elevated [Ca2+] and hence slowing relaxation. The elevation in [Ca2+]i leads to formation of the Ca-calmodulin complex which then activates MLC kinase, resulting in acto-myosin crossbridge cycling and contraction.

DAG activation of PKC activates the MAPK cascade resulting in increased PLA2 activity and prostaglandin production, which also contributes to contraction. DAG-activated PKC also signals to activate ROCK. ROCK inhibits MLC phosphatase leading to increased MLC phosphorylation and increased force (Ca2+ sensitization). Hypoxia also lowers ATP and stimulates opening plasma membrane KATP channels, resulting in hyperpolarization. This will relax smooth muscles, and conditioning can be elicited using KATP channel agonists.

During hypoxia HIF levels accumulate and lead to many of the metabolic and gene expression changes integral to conditioning. This includes effects on mitochondrial metabolism. The mitochondrial ATP channel is activated by conditioning and helps prevent opening of the mitochondrial permeability transition pore.

Nitric oxide is elevated by a variety of mechanisms in conditioning, including adenosine activation, elevation of cGMP and stimulation of eNOS in caveolae.

VOCC: voltage operated Ca2+ channel, PLC-β: phospholipase C-β, PIP2: phosphatidylinositol 4,5-bisphosphate, IP3: inositol 1,4,5- triphosphate, DAG: diacylglycerol, PKC: protein kinases type C, Ca-CAM: Ca-calmodulin complex, MLCK: myosin light-chain kinase, MLCP: myosin light chain phosphatase, MAPK: mitogen-activated protein kinase, PLA2: phospholipase A2, ROCK: RhoA-associated protein kinase, NO: nitric oxide’ ROS: reactive oxygen species, KATP and m KATP: plasma membrane and mitochondrial ATP-sensitive K channels, respectively’ mPTP: mitochondrial permeability transition pore, HIF: hypoxia-inducible factor.

Adapted from Arrowsmith S & Wray S (7)

**Figure 2: External factors contributing to myocyte conditioning.**

The smooth muscle myocyte (central cell) can be conditioned by nervous activity, products of inflammation such as cytokines, immune cells and responses, humoral and paracrine factors, and the endothelial or epithelial cells in the tissues the myocytes reside.

Based in part on Meller R & Simon RP (123)

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