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Inducible Endothelium-Derived Hyperpolarizing Factor (iEDHF): Role of the 15-Lipoxygenase-EDHF Pathway

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Abstract

Endothelium-derived hyperpolarizing factors (EDHFs) regulate vascular tone by contributing to the vasorelaxations to shear stress and endothelial agonists such as bradykinin and acetylcholine. 15(S)-Hydroxy-11,12-epoxyeicosatrienoic acid (15-H-11,12-EETA) and 11(R),12(S),15(S)-trihydroxyeicosatrienoic acid (11,12,15-THETA) are endothelial metabolites of the 15-lipoxygenase (15-LO) pathway of arachidonic acid metabolism and are EDHFs. 11,12,15-THETA activates small conductance, calcium-activated potassium channels on smooth muscle cells causing membrane hyperpolarization and relaxation. Expression levels of 15-LO in the endothelium regulate the activity of the 15-LO/15-H-11,12-EETA/11,12,15-THETA pathway and its contribution to vascular tone. Regulation of its expression is by transcriptional, translational and epigenetic mechanisms. Hypoxia, hypercholesterolemia, atherosclerosis, anemia, estrogen, interleukins and possibly other hormones increase 15-LO expression. An increase in 15-LO results in increased synthesis of 15-H-11,12-EETA and 11,12,15-THETA, increased membrane hyperpolarization and enhanced contribution to relaxation by endothelial agonists. Thus, the 15-LO pathway represents the first example of an inducible EDHF. In addition to 15-LO metabolites, a number of chemicals have been identified as EDHFs and their contributions to vascular tone vary with species and vascular bed. The reason for multiple EDHFs has evaded explanation. However, EDHFs functioning as constitutive EDHFs or inducible EDHFs may explain the need for chemically and biochemically distinct pathways for EDHF activity and the variation in EDHFs between species and vascular beds. This new EDHF classification provides a framework for understanding EDHF activity in physiological and pathological conditions.

Keywords

Endothelium-derived hyperpolarizing factor; 15-Lipoxygenase; vascular relaxation; potassium channels; THETA; HEETA

Endothelial cells regulate vascular tone through the release of several soluble mediators [1–4]. These mediators include nitric oxide (NO), prostaglandin I2 (PGI_2) and endothelium-derived hyperpolarizing factors (EDHFs). They are released by a number of stimuli including endothelial agonists such as acetylcholine and bradykinin and shear stress and mediate vasorelaxation. When the synthesis of NO and PGI_2 are inhibited by NO synthase (NOS) and cyclooxygenase (COX) blockers, a component of the endothelium-dependent relaxations to acetylcholine or bradykinin persists and is associated with hyperpolarization of the smooth muscle cell membrane [2–4]. These activities are attributed to EDHF. Interestingly, the contribution of EDHF to vascular tone is more prominent in resistance

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arteries than conduit arteries [5]. Unlike NO and PGI_2, EDHF is not a single chemical compound but a family of endothelial factors that hyperpolarize and relax smooth muscle. Several endogenous compounds mediate endothelium-dependent hyperpolarization in various arteries and species (Figure 1). These include metabolites of arachidonic acid (AA) such as epoxyeicosatrienoic acids (EETs), 15-hydroxy-11,12-epoxyeicosatrienoic acids (15-H-11,12-EETA) and 11,12,15-trihdyroxyeicosatrienoic acid (THETA), potassium (K) ion, hydrogen peroxide and C-type natriuretic peptide (CNP)(Figure 1)[3, 4, 6–11]. Despite differences in their chemical structure, these EDHFs act on vascular smooth muscle cells to activate calcium-activated K (K_{Ca}) channels or inward rectifying K (K_{ir}) channels to cause membrane hyperpolarization and inhibition of calcium influx through voltage-activated calcium channels resulting in vasorelaxation. Gap junctions between endothelial cells, smooth muscle cells and the two cell types transmit the hyperpolarization along the vascular wall. Alternatively, endothelium-dependent hyperpolarization may not require a soluble mediator [4, 8, 12, 13]. Acetylcholine stimulates calcium influx into endothelial cells activating K_{Ca} channels and causing the membrane of endothelial cells to hyperpolarize. Myo-endothelial gap junctions transfer the endothelial hyperpolarization to the smooth muscle cells resulting in relaxation. Since a factor is not involved, this mechanism has been called endothelium-dependent hyperpolarizations (EDH). Thus, endothelium-dependent hyperpolarization may occur by the transfer of a soluble factor (EDHF) or the transfer of hyperpolarization between endothelial cells and smooth muscle cells (EDH).

Regulation of the synthesis and release of endothelial factors

In addition to acetylcholine, bradykinin and shear stress, endothelial mediators of vasorelaxation are regulated in other ways. NO is synthesized from L-arginine by NOS. There are three NOS isozymes: endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS)[14]. Of these enzymes, eNOS and nNOS are constitutively expressed in various cells and tissues while the expression of iNOS is regulated. Similarly, there are two isoforms of COX involved in the synthesis of PGs including PGI_2 [15]. COX-1 is constitutively expressed in most cells whereas COX-2 is an inducible form of the enzyme. Cytokines and growth factors induce the expression of both COX-2 and iNOS. It would seem logical that analogous dual regulatory pathways exist for EDHF. We present evidence in this review that constitutive EDHF (cEDHF) or cEDH and inducible EDHF (iEDHF) exist in arteries. A cEDHF is expressed in the endothelium of most blood vessels under basal conditions, is released by endothelial agonists and mediates a portion of endothelium-dependent relaxation; however, the chemical or electrical nature may vary with vascular beds and with species. iEDHF is dormant under normal circumstances so does not contribute to endothelial agonist-stimulated relaxations. However, iEDHF is induced under various physiological and pathological conditions to participate in endothelium-dependent relaxation by endothelial agonists. Thus, the contribution to iEDHF to endothelium-dependent relaxation may be absent under basal conditions but its contribution is enhanced when induced. While both COX-1 and COX-2 produce PGI_2 and eNOS, nNOS and iNOS produce NO, cEDHF and iEDHF are different chemical entities or chemical and electrical entities. Since cEDHF and iEDHF may activate different K channels, a synergistic or additive interaction is possible and may result in greater vasodilation with their combination.

Classification of EDHFs

The EDHFs can be divided into cEDHFs and iEDHFs based on our current knowledge (Figure 1)(The shaded numbers in Figure 1 refer to the corresponding numbered descriptions of major EDHFs below.):

1. Activation of endothelial intermediate conductance K_{Ca} (IK_{Ca}) and small conductance K_{Ca} (SK_{Ca}) channels hyperpolarizes endothelial cells and releases K

ion into the sub-endothelial space [8, 10, 12, 13]. This results in hyperpolarization of smooth muscle cells through gap junctional transfer of the hyperpolarization (EDH) and/or K ion activating K_{ir} channels or the sodium-potassium ATPase on smooth muscle cells (EDHF). This EDH or EDHF is constitutive in several vascular beds. There is no current evidence of enhanced expression of its components.

2. Several EDHFs are constitutively expressed; however, their synthesis or expression may also increase. For example, CNP is constitutively expressed in endothelial cells, is released by endothelial agonists and relaxes smooth muscle by increasing cyclic GMP and membrane hyperpolarization [9]. The endothelial expression and secretion of CNP is increased by transforming growth factor-β (TGF-β), tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1)[16–18]. In some, but not all, reports, platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) increase CNP expression in smooth muscle cells. The enhanced secretion of CNP with these cytokines and growth factors is regulated by its synthesis and not endothelial agonists.

3. Hydrogen peroxide is formed by hydration of superoxide by superoxide dismutase [19, 20]. It is constitutively present in some arteries and mediates a portion of the relaxation to endothelial agonists. The enzymatic source of superoxide varies with vascular bed. For example, superoxide may arise from NADPH oxidase, mitochondria or NOS [20–22]. Although not studied in detail, the superoxide generating enzyme(s) rather than superoxide dismutase appears to be rate limiting in vascular hydrogen peroxide formation. The three NOS isozymes are sources of vascular superoxide and hydrogen peroxide in the mouse mesenteric artery [20]. Thus, eNOS and nNOS represent constitutive sources of hydrogen peroxide and iNOS as an inducible source. However, iNOS is not regulated by endothelial agonists.

4. EETs are cytochrome P450 (CYP) metabolites of AA [23, 24]. CYPs are a large family of enzymes with subsets that metabolize xenobiotics and drugs, synthesize steroid hormones and metabolize fatty acids. Several CYPs are constitutively expressed in endothelial cells including CYP2C and CYP2J. The EETs are synthesized by the endothelium and released by acetylcholine, bradykinin and shear stress [4, 6, 25]. They hyperpolarize and relax vascular smooth muscle by activating large conductance K_{Ca} (BK_{Ca}) channels. Drugs, xenobiotics and hypoxia increase the expression of certain CYP epoxygenases and increase EET-mediated hyperpolarization and relaxation [26–29]. Inflammatory cytokines reduce the expression and activity of CYP epoxygenases by increasing iNOS and NO synthesis [30]. Thus, EETs function as cEDHFs; however, the synthesis of endothelial EETs may be increased by conditions or treatments that increase the expression of CYP epoxygenases.

5. 15-H-11,12-EETA and 11,12,15-THETA are endothelial 15-lipoxygenase (15-LO) metabolites of AA [11, 31]. They mediate a portion of the relaxations to acetylcholine in several arteries by activating smooth muscle cell SK_{Ca}-like channels and causing hyperpolarization. They may also act through other mechanisms. Basal expression of 15-LO is elevated in arteries of neonatal and young animals but is absent in adolescent and adult animals [32, 33]. However, the expression of 15-LO is increased by cytokines, estrogen, hypoxia and hypercholesterolemia resulting in enhanced production of HEETA/THETAs and enhanced relaxations to acetylcholine [34–37]. The 15-LO/HEETA/THETA pathway is an iEDHF and will be examined in more detail.

Role of LO metabolites of AA as EDHFs

Furchgott and Zawadzki first showed that endothelium-dependent relaxations to acetylcholine were mediated by a LO metabolite of AA [38]. In the rabbit aorta, LO inhibitors and inhibitors of AA release by phospholipase A2 attenuated the relaxations to acetylcholine. COX inhibitors were without effect. Using the same pharmacological approach, a role for LO metabolites in the endothelium-dependent relaxations to acetylcholine, bradykinin, histamine, thrombin, substance P and other agonists was demonstrated in arteries from a variety of species and vascular beds [39–47]. When the synthesis of NO and PGI_2 are inhibited, the relaxations to acetylcholine are inhibited, but not blocked, indicating a component of the relaxations is mediated by EDHF [11, 31, 47]. A role for EDHF was confirmed by showing that the residual relaxations to acetylcholine were inhibited by high extracellular K that blocks K channels and by the SK_{Ca} channel inhibitor, apamin. These relaxations are also inhibited by LO inhibitors. When antisense oligonucleotides were used to suppress the expression of 15-LO in rabbit aorta, the non-NO, non-PGI_2-mediated relaxations to acetylcholine were inhibited [48]. Scrambled oligonucleotides that did not affect 15-LO expression did not alter the relaxations to acetylcholine. These studies support a role for LO metabolites as EDHFs causing endothelium-dependent relaxations through activation of apamin-sensitive K channels. These K channels have properties of SK_{Ca} channels [49]; however, SK_{Ca} channels are localized to the endothelium rather than smooth muscle so the term SK_{Ca}-like channels will be used [13].

Additionally, AA caused endothelium-dependent relaxations in a number of species and arteries [34, 39, 50–55]. These relaxations were inhibited by LO inhibitors and enhanced by COX inhibitors. Inhibition of COX provided more AA for metabolism to vasoactive metabolites by LO [34]. As with acetylcholine, the relaxations to AA were inhibited by high extracellular K and by apamin indicating a role for SK_{Ca}-like channels in the relaxations [49]. In rabbit aorta, AA also hyperpolarized smooth muscle cells if the endothelium was intact but not when the endothelium was removed [31, 49, 56]. Endothelium-dependent hyperpolarizations to AA were inhibited with the SK_{Ca} channel inhibitor, apamin. Thus, like acetylcholine, endothelium-dependent relaxations to AA are mediated by LO metabolites that activate apamin-sensitive SK_{Ca}-like channels, to cause smooth muscle membrane hyperpolarization and relaxation. These studies with acetylcholine and AA support a role for LO metabolites as EDHFs.

Endothelial metabolism of AA by 15-LO

LOs are a family of non-heme containing oxygenases that add molecular oxygen at double bonds of polyunsaturated fatty acids such as AA [57–59]. In the case of AA, the LO product is a hydroperoxyeicosatetraenoic acid (HPETE) that is reduced by glutathione peroxidase to a hydroxyeicosatetraenoic acid (HETE). The LOs are named for the position of the hydroperoxy or hydroxy group on AA. In most cases, the vascular LOs were identified by immunoblotting or PCR. However, the rabbit aortic LO was cloned, sequenced and identified as 15-LO-1 48. It is localized to the endothelium. The two main LOs in arteries and endothelial cells of humans, dogs, pigs, cows and rabbits are 12-LO and 15-LO [11]. 12-LO synthesizes predominately 12-HPETE, and 15-LO synthesizes predominately 15-HPETE [58, 59]. The major vasoactive LO metabolites arise from 15-LO [11]. Rats and mice have a single LO that synthesizes both 12- and 15-HPETE [60, 61]. The rodent 12/15-LOs synthesize 12-HPETE in a three-fold greater amount than 15-HPETE. Thus, there are clear species differences in the LOs and AA products from vascular LOs. The vascular AA metabolites from rodent 12/15-LO differ sufficiently from human 15-LO that they may not be comparable.

The vascular metabolism of AA by 15-LO is outlined in Figure 2. The endothelium is the major source of AA metabolites. Agonists such as acetylcholine or bradykinin regulate AA release from membrane phospholipids by activating either phospholipase A2 or the phospholipase C pathway [62, 63]. Free AA is metabolized by 15-LO to 15(S)-HPETE [64]. Two competing pathways metabolize 15(S)-HPETE. Reduction of 15(S)-HPETE by glutathione peroxidase produces 15(S)-HETE that has little or no vasoactivity. Alternatively, 15(S)-HPETE is rearranged by a hydroperoxide isomerase to 15-H-11,12-EETA [56, 64]. The epoxy group is unstable in acid with a half-life of less than 10 s at pH 3 but 33 h at pH 7.4. Hydration of the epoxide also occurs enzymatically in arteries. 15-H-11,12-EETA is metabolized by soluble epoxide hydrolase (sEH) to 11,12,15-THETA [56]. With three hydroxyl groups, many stereoisomers of 11,12,15-THETA are possible. However, of the possible isomers, only 11(R),12(S),15(S)-THETA comigrated with the 11,12,15-THETA produced by the aorta [65]. The stereochemical configuration of endogenous 15-H-11,12-EETA is not known. Both 15-H-11,12-EETA and 11(R),12(S),15(S)-THETA cause relaxation [56, 65]. In contrast, AA is metabolized by 12-LO to 12-HPETE and 12-HETE [57]. 12-HETE is also the major metabolite of rodent 12/15-LO [60]. 12-HETE relaxes some arteries but not others [66, 67]. There is currently no evidence for the synthesis of other vasoactive metabolites from 12-HPETE by arteries.

Mechanism of action of endothelial 15-LO metabolites

As indicated above, AA causes endothelium-dependent hyperpolarizations and relaxations that are inhibited by blocking K channels with high extracellular K and by the SK_{Ca} channel inhibitor apamin but not by the IK_{Ca}/BK_{Ca} channel inhibitor charybdotoxin [31, 49]. Aortic smooth muscle cells have a 24 pS K channel that is activated by calcium and inhibited by apamin. This is consistent with the presence of a SK_{Ca}-like channel. These findings suggest that AA is metabolized to a LO metabolite(s) that activate smooth muscle SK_{Ca} channels causing membrane hyperpolarization and relaxation. 15-H-11,12-EETA and 11,12,15-THETA as the major endothelial 15-LO metabolites would be expected to act by this same mechanism. Due to its instability, 15-H-11,12-EETA has not been tested directly on arteries. However, inhibition of the metabolism of 15-H-11,12-EETA with a sEH inhibitor enhances the relaxations to acetylcholine [56]. Inhibition of sEH also enhances the endothelium-dependent hyperpolarization and relaxation to AA. These enhanced hyperpolarizations and relaxations to AA are blocked by LO inhibition. These indirect studies indicate that 15-H-11,12-EETA causes smooth muscle hyperpolarization and vasorelaxation.

11,12,15-THETA also causes relaxation of rabbit and mouse arteries [31, 65]. This vasoactivity is limited to the specific 11,12,15-THETA stereoisomer that is produced by the vascular endothelium. 11(R),12(S),15(S)-THETA relaxes rabbit arteries while seven other stereoisomers are inactive [65]. 11(R),12(S),15(S)-THETA also increases the activity of an apamin-sensitive K channel in aortic smooth muscle cells. The finding that a specific stereoisomer of 11,12,15-THETA activates smooth muscle SK_{Ca}-like channels and causes relaxation indicates that a specific binding site or receptor must mediate these effects. This binding site/receptor has not been characterized further.

Regulation of Vascular 15-LO

While the release of AA from membrane lipids by agonists is essential for 15-LO synthesis of eicosanoids, 15-LO is the key enzyme in regulating the activity of this pathway and regulating its contribution to vascular tone. Thus, variation in the expression of 15-LO is an important determinant of the pathway's role in physiological and pathological conditions. Increasing the expression of 15-LO enhances endothelium-dependent relaxations whereas inhibition of 15-LO has the opposite effect. For example, rabbit arteries were transduced

with a 15-LO-containing adenovirus to increase the endothelial expression of 15-LO [68, 69]. In transduced arteries, the increased expression of endothelial 15-LO increased the synthesis of the 15-LO metabolites, 15-H-11,12-EETA and 11,12,15-THETA and the relaxations to acetylcholine and AA when compared to non-transduced arteries. Pharmacological inhibition of 15-LO activity or decreasing endothelial 15-LO expression with 15-LO antisense oligonucleotides reduced the synthesis of the vasoactive AA metabolites and inhibited relaxations to AA and acetylcholine [31, 48, 50, 65]. Thus, variations in the expression of 15-LO are important to the endothelium-dependent regulation of vascular tone and vascular EDHF activity.

The expression of 15-LO is regulated at several levels: epigenetic modifications, transcription and translation [70, 71]. Transcription is stimulated by cytokines such as interleukin (IL)-4 and IL-13 through activation of the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) transcription factor pathway or the p38 mitogen-activated protein kinase (MAPK)-STAT or protein kinase C (PKC)-STAT pathways [72, 73]. Steroid hormone receptors and hormone response elements, Ku70/80 and GATA transcription factors and methylation and acetylation of the 15-LO promoter region also regulate 15-LO transcription [71, 74, 75]. The 3′-untranslated region of the 15-LO mRNA contains a differentiation control element [76]. When the translational repressor proteins heterogeneous nuclear ribonucleoproteins (hnRNP)-E1 or -K are bound to this element, translation of the 15-LO mRNA is prevented. Activities of hnRNP proteins are regulated by phosphorylation [77]. When phosphorylated by MAP kinase or src kinase, hnRNP localizes to the cytosol, rather than the nucleus, so translation is inhibited [78]. Growth factors and agonist acting on G-protein coupled receptors may regulate 15-LO mRNA transcription through src kinase- or MAP kinase-hnRNP pathway. Thus, there are multiple regulators of 15-LO expression and many mechanisms of regulation.

Conditions and endogenous chemicals regulating 15-LO and iEDHF activity

A number of pathological conditions and endogenous compounds induce the expression of 15-LO. While there are many studies on the regulation of 15-LO in cultured cell lines or inflammatory cells, investigations in endothelial cells, smooth muscle cells and arteries are more limited. In arteries, the increase in endothelial 15-LO is accompanied by an increase in the synthesis of vasodilatory eicosanoids, smooth muscle membrane hyperpolarization and enhanced endothelium-dependent relaxations [68, 69].

1. Changes in 15-LO with age

EDHF activity decreases with age in rodents and rabbits [32, 33, 79]. 15-LO expression was very high in aortas from 1 week old rabbits and declined by 10% at 4 weeks and 40% by 8 weeks of age [32, 33]. This was paralleled by a decline in the synthesis of AA metabolites of 15-LO by aortic and mesenteric rings over the same time period. In the presence of a COX inhibitor, the major AA metabolites were THETAs, HEETAs and HETEs and the synthesis of these metabolites were blocked by the LO inhibitor BW755C. At 8 weeks of age, the synthesis of HEETA, THETA and HETEs were not detectable. The EDHF-mediated relaxations to acetylcholine were reduced in aortas and mesenteric arteries of 4, 8 and 16 week old rabbits compared to 1 week old rabbits [32]. Maximal relaxations to acetylcholine were reduced by approximately 50% in arteries from 16 week old rabbits compared to 1 week old rabbits. Responses to AA were similarly reduced with age whereas endothelium-independent relaxations to sodium nitroprusside were not changed. In arteries from 1 week old rabbits, EDHF-mediated relaxations to acetylcholine were inhibited by 40–50% by either LO inhibition by BW755C or IK_{Ca}/BK_{Ca} channel inhibition by charybdotoxin [33]. The combination of BW755C and charybdotoxin blocked the relaxations. Thus, a component is mediated by 15-LO and HEETA and THETA and a component by IK_{Ca} channels and K ion.

In contrast, LO inhibition did not alter the relaxations to acetylcholine in aortas from 16 week old rabbits whereas IK_{Ca}/BK_{Ca} channel inhibition blocked the relaxations. Similar results were obtained in vivo when blood pressure decreases to acetylcholine were measured [33]. Thus, at birth, vascular 15-LO expression is high and 15-LO metabolites contribute to endothelium-dependent dilation. The mechanism responsible for the increased expression is not known. The 15-LO-mediated component of EDHF is lost in the months following birth while the IK_{Ca} component is unchanged and becomes the sole mediator. These findings are consistent with the 15-LO pathway functioning as an iEDHF and the IK_{Ca}-K ion pathway functioning as cEDHF. iEDHF and cEDHF function together in early life to maintain dilation and organ perfusion. Over the next months, the stimulus inducing 15-LO expression is reduced so the pathway becomes dormant and cEDHF mediates endothelium-dependent relaxations.

2. Regulation of 15-LO by hypoxia

Hypoxia induced the expression of 15-LO in rabbit lung microsomes when compared to normoxia 36. In contrast, 12-LO was unchanged. 15-LO was localized in the lung to pulmonary arteries. Lung microsomes synthesized THETAs, HEETAs and 15-HETE, and the synthesis of these AA metabolites was 3-fold greater with hypoxia than normoxia. 15-HETE synthesis was blocked by LO inhibition but not by COX or CYP inhibitors. Hypoxia also increased the expression of 15-LO-1 and the synthesis of 15-HETE compared to normoxia in human microvascular endothelial cells [80]. Thus, both in vivo and in vitro hypoxic conditions increase vascular 15-LO. The mechanism for hypoxia inducing 15-LO expression is not known. The promoter region of 15-LO does not contain the hypoxia response element suggesting that hypoxia acts indirectly to induce the enzyme, possibly through platelet-derived growth factor (PDGF)[81].

In rabbit aortic endothelial cells, hypoxia increased the metabolism of AA by 15-LO. The synthesis of THETAs, HEETAs and HETEs was greater in cells exposed to hypoxic compared to normoxic conditions (Figure 3). The membrane potential did not differ in aortic smooth muscle cells of normoxic or hypoxic rabbits. However, hyperpolarization to AA was enhanced in smooth muscle cells of hypoxic rabbits. The acetylcholine-induced relaxations mediated by EDHF were also greater in arterial rings from hypoxic compared to normoxic rabbits. Thus, hypoxia induces the expression of 15-LO resulting in the increased synthesis of THETA and HEETA and increased hyperpolarization and relaxation. Hypoxia activates this iEDHF.

3. Regulation of 15-LO by cholesterol, anemia and atherosclerosis

The expression of 15-LO is increased in atherosclerotic lesions in arteries of rabbits and human [82, 83]. The synthesis of THETAs, HEETAs and HETEs was increased in aortas of rabbits fed a high cholesterol diet for 2 weeks compared to a normal diet [34]. The synthesis of the THETAs was inhibited by LO inhibitors but enhanced by COX inhibition. Hypercholesterolemia and elevated 15-LO were associated with a 40% lowering of the blood hematocrit [84]. Lowering the hematocrit by other means and experimental anemia increase 15-LO expression [70, 84].

In addition to the increase in 15-LO expression, relaxations to AA were enhanced in aortas of cholesterol-fed rabbits compared to controls [34]. Similarly, the relaxations were enhanced by COX inhibition and reduced by LO inhibition or removal of the endothelium. While the membrane potential was unchanged in smooth muscle cells of aortas from normal and cholesterol-fed rabbits, hyperpolarization to AA was greater in smooth muscle of cholesterol-fed rabbits (Figure 4). The EDHF component of relaxation is also greater with cholesterol feeding. In NOS- and COX-inhibited aortic rings, acetylcholine relaxations were

enhanced in rings from cholesterol-fed rabbits compared to rabbits on a normal diet. Thus, the 15-LO component of EDHF is increased with hypercholesterolemia.

4. Regulation of 15-LO by interleukins

Both IL-4 and IL-13 stimulate 15-LO expression in a variety of cell types [35, 73, 85]. As indicated above, IL-4 and IL-13 activate transcription of the 15-LO gene through the JAK-STAT, src-STAT or PKC-STAT pathways [73]. Transgenic mice overexpressing IL-4 show increased 15-LO activity in the lung, spleen, kidney and heart [72]. IL-4 induced the transcription of the 15-LO-1 gene in human endothelial cells; however, an increase in the expression of the 15-LO-1 enzyme was not detected [85]. The reason for this dissociation between gene activation and protein expression is not known. In monocytes and other cell types, IL-4 increases 15-LO-1 mRNA, protein and AA metabolites [72]. IL-13 increased the transcription of the 15-LO-1 gene and expression of the 15-LO-1 protein in rabbit aortic endothelial cells and aortic rings [35]. In aortic rings, 15-LO was localized predominately to the endothelium. Following IL-13 treatment, immunohistochemical staining for 15-LO markedly increased in the endothelium and in smooth muscle cell layers next to the endothelium. Metabolism of AA to THETAs, HEETAs and HETEs by aortic rings was greater in IL-13-treated rings compared to control rings. Similarly, relaxations to AA were enhanced in rings treated with IL-13 compared to untreated rings. These studies indicate that the 15-LO-EDHF pathway is induced by Th2 cytokines, IL-4 and IL-13.

5. Regulation of 15-LO by estrogen

Gender and estrogen clearly influence EDHF activity. The EDHF-mediated relaxations to acetylcholine in femoral arteries were greater in female than male mice [86]. This difference was absent in female mice lacking the estrogen receptor. Acetylcholine-induced relaxations that are mediated by EDHF were greater in estrogen-treated male rats than control rats [87]. When estrogen deficiency is produced by ovariectomy of female rats, the opposite effect is observed [88]. Ovariectomy reduced EDHF-mediated relaxations in mesenteric arteries. Estrogen treatment of the ovariectomized rats restored the EDHF component to relaxation.

The expression of 15-LO and 5-LO, but not 12-LO, increased in pulmonary arteries of female rabbits compared to male rabbits [37]. This was associated with an increased metabolism of AA to 15-HETE and 5-HETE by arteries from females compared to males. When male pulmonary arteries were treated with estrogen, the expression of 15-LO was increased in estrogen-treated arteries compared with control arteries. Additionally, the metabolism of AA to THETAs, HEETAs and HETEs was enhanced in estrogen-treated pulmonary arteries. EDHF activity was not evaluated in these arteries. Estrogen treatment of human endothelial cells also increased the expression of 15-LO by three-fold [89]. These studies indicate that estrogen increases the expression of the 15-LO pathway in arteries and endothelial cells.

6. Other regulators of 15-LO

A number of other endogenous mediators increase the cellular expression of 15-LO; however, these mediators were tested in cultured cells and not in endothelial cells or arteries. Thus, their ability to induce the endothelial 15-LO pathway of iEDHF is in need of investigation. For example, angiotensin II, aldosterone, PDGF, TGF-β, IL-8, growth hormone-releasing peptide-2, peroxisome proliferator-activated receptor-γ agonist and high glucose increase 15-LO expression or rodent 12/15-LO expression in cultured smooth muscle cells or mesangial cells when compared with untreated cells [90–96]. 15-LO expression is also increased in human umbilical arteries from patients with pre-eclampsia compared to normal patients [97].

In summary, many conditions, cytokines, growth factors and hormones regulate 15-LO and/or 12/15-LO expression in vascular and non-vascular cells; however, their role in regulating iEDHF activity has not been investigated. Clearly, age, hypoxia, hypercholesterolemia and atherosclerosis, Th2 cytokines and estrogen increase the expression of 15-LO and enhance the synthesis of 15-LO-derived vasodilatory eicosanoids, smooth muscle cell hyperpolarization and EDHF-mediated relaxation. Thus, these conditions and mediators induce 15-LO-mediated iEDHF activity.

Interactions between iEDHF and other endothelial mediators

PGI_2, NO, cEDHF and iEDHF may be co-release from the endothelium by endothelial agonists. Thus, interactions between these endothelial mediators in regulating vascular tone are important. COX and 15-LO complete for the same substrate, AA. As a result, when COX was inhibited, more AA was available for metabolism by 15-LO and the synthesis of 11,12,15-THETA and 15-H-11,12-EETA increased [34]. In rabbit mesenteric arteries, relaxations to AA were partially inhibited by COX inhibition and blocked by the combination of COX and LO inhibition suggesting near equal contributions to relaxation by COX and 15-LO metabolites [54]. The effect of PGI_2 on relaxations to 11,12,15-THETA or 15-H-11,12-EETA and visa versa has not been determined. The relaxations to acetylcholine were significantly inhibited by LO and SK_{Ca}-like channel inhibition but not by COX inhibition. Thus, the 15-LO pathway of AA metabolism contributes more to acetylcholine-induced relaxations than the COX pathway. The reason for the difference in the contributions of these pathways to the relaxations to AA and acetylcholine is not known.

The interaction between the 15-LO and NOS pathways has been investigated [98]. The NO donor DPTA-NONOate relaxed the rabbit aorta, and the relaxations were blocked by the soluble guanylyl cyclase inhibitor ODQ but not by LO inhibition. The relaxations to acetylcholine were inhibited by ODQ, NOS inhibition or LO inhibition and blocked by the combination of ODQ and LO inhibition. Thus, the NOS and LO pathways mediate the relaxations to acetylcholine. In contrast, ODQ did not alter relaxations to AA whereas the combination of COX and LO inhibition blocked the relaxations. Consistent with this finding, the metabolism of AA to THETA, HEETA and 15-HETE by rabbit aorta was not altered by NOS inhibition or by physiological concentrations of DPTA-NONOate. When endogenous NO synthesis was inhibited, DPTA-NONOate was added to restore the NO contribution. The amount of relaxation to acetylcholine was the same in the presence or absence of DPTA-NONOate. Thus, the NOS and 15-LO pathways act in parallel to mediate the relaxations to acetylcholine. Also, NO does not alter the activity of the 15-LO pathway.

iEDHF enhanced relaxations mediated by cEDHF (Figure 5)[55]. In rabbit mesenteric arteries, acetylcholine stimulated the endothelial release of THETAs and voltage-dependent outward K currents from endothelial cells. Thus, THETAs and K ion are co-released from the endothelium by acetylcholine. In the presence of COX and NOS blockade, inhibition of the synthesis or action of the THETA with a LO inhibitor or SK_{Ca} channel inhibitor partially inhibited the relaxations to acetylcholine as did inhibition of K release or K action with an IK_{Ca}/BK_{Ca} channel inhibitor or K_{ir} channel inhibitor. Combining inhibitors of both pathways blocked the relaxations of acetylcholine indicating their combined participation in the relaxations. Addition of 10.9 mM K ion potentiated the relaxations to both AA and 11,12,15-THETA. Thus, acetylcholine-induced, EDHF-dependent relaxation of mesenteric arteries involves two separate and parallel mechanisms: K ion, a cEDHF and 11,12,15-THETA, an iEDHF.

Role of EDHF/EDH in the endothelial dysfunction of pathological conditions

Many cardiovascular diseases such as hypertension, diabetes, ischemic heart disease, renal failure and congestive heart failure are associated with reductions in endothelium-dependent relaxations allowing endothelium-dependent constrictors or circulating constrictors to act unopposed [99]. This has been termed endothelial dysfunction and is thought to contribute to the pathology of these diseases. Correction of endothelial dysfunction may represent a new therapeutic approach. The diminished endothelium-dependent relaxations may be due to a reduced contribution of NO and/or EDHF. The role(s) of specific EDHFs or EDH in endothelial dysfunction may vary in specific diseases but comprehensive investigations are lacking.

1. Hypertension

In isolated arteries from renal or spontaneously hypertensive rats, endothelium-dependent relaxations and hyperpolarizations to acetylcholine were reduced [100–103]. The contributions of both NO and EDHF/EDH were diminished. In spontaneously hypertensive rats, the cEDHF/EDH pathway mediated by potassium ion was reduced [103]. While the contribution by endothelial IK_{Ca} channels was unaltered, the contributions of both SK_{Ca} and K_{ir} channels were reduced. The iEDHF pathway was not investigated; however, iEDHF did not compensate for the reduction of cEDHF to maintain endothelial function. Endothelium-dependent dilation was also decreased in patients with essential hypertension [104–106]. Measuring endothelium-dependent dilation by forearm blood flow, the NO contribution to dilation was lost in hypertensive patients; however, the EDHF component mediated by EETs was retained and partially compensated for the loss of NO [104]. In contrast, both the NO and EET components were reduced in hypertensive patients when endothelium-dependent dilation was measured by changes in radial artery diameter 105, [106]. The 15-LO iEDHF pathway has not been assessed in essential hypertension. Clearly, in hypertension, cEDHF and iEDHF do not compensate for the loss of NO so endothelial dysfunction ensues.

2. Diabetes mellitus and insulin resistance

Endothelium-dependent dilations and hyperpolarizations were reduced in arteries from rats with streptozotocin-induced diabetes and in Zucker diabetic fatty rats [107–110]. Experimental studies differ regarding whether reductions in NO and EDHF or only EDHF are responsible for the endothelial dysfunction. The relaxations and hyperpolarizations to IK_{Ca} channel activation with EBIO or IK_{Ca} and SK_{Ca} channel activation with NS309 were reduced in diabetic rats [108, 109]. Thus, the reduction in EDHF/EDH in diabetes may be attributed to attenuation of K channels of the cEDHF pathway. The iEDHF pathway has not been investigated. However, as with hypertension, iEDHF does not prevent the endothelial dysfunction of diabetes.

Endothelium-dependent relaxations were also reduced in insulin-resistant rats [111]. The EDHF-mediated relaxations were inhibited by the cytochrome P450 inhibitor miconazole in normal rats, but miconazole was without effect in insulin-resistant rats. Induction of cytochrome P450 with 14-days of pentobarbital treatment increased the EDHF-mediated relaxations in insulin-resistant rats and improved endothelial dysfunction. Phenobarbital treatment also reduced blood pressure in insulin-resistant rats. The 15-LO iEDHF pathway was not studied, and the effect of phenobarbital on 15-LO is not known. Under these experimental conditions, induction of cytochrome P450-derived EETs restores endothelial function in insulin-resistant rats.

3. Congestive heart failure and ischemic preconditioning

Congestive heart failure (CHF) was induced in rats by left coronary artery ligation [112, 113]. Endothelium-dependent relaxations did not differ in arteries of control and CHF rats. However, the EDHF-mediated contribution was increased, and the NO-mediated contribution was reduced. Upregulation of EDHF compensated for the loss of NO. The EDHF pathway, cEDHF or iEDHF, that increased in CHF was not determined. Similar results were obtained in patients with CHF [114]. Increases in forearm blood flow with acetylcholine were the same in normal subjects and CHF patients. Inhibition of NO synthesis reduced the dilation to acetylcholine in normal subjects but had no effect in CHF patients. Thus, EDHF is the major mediator of endothelium-dependent dilation in CHF patients. The EDHF mediating dilation in CHF is not known.

Similarly, in mice, ischemia-reperfusion injury reduced the endothelium-dependent relaxations to acetylcholine [115]. A reduction in the NO-mediated component was responsible. The EDHF component was increased. Hypoxic pre-conditioning enhanced both the NO- and EDHF-mediated relaxations and eliminated the endothelial dysfunction. The expression of endothelial TRPV4 and connexins and phosphorylation of eNOS increased with preconditioning. The increase in TRPV4 resulted in increased intracellular calcium in endothelial cells that activates eNOS and K_{Ca} channels. Increases in connexins enhance gap junctions to increase EDH and the spread of the EDHF response along the artery. Hypoxia increases the expression of 15-LO; however, the role of 15-LO iEDHF was not determined.

In summary, endothelial dysfunction is observed in many cardiovascular diseases. In some cases such as hypertension and diabetes, EDHF is impaired and unable to compensate for a loss of NO so endothelium-dependent dilation is reduced. However, in other conditions such as CHF, EDHF compensates for the loss of NO so endothelial function is normal. The role of cEDHF and iEDHF in these diseases merits further study.

Conclusion

The endothelium releases a number of chemicals that mediate endothelium-dependent hyperpolarization. The reasons for redundancy are not apparent. Likely, the EDHFs serve different physiological and/or pathological functions. We suggest that some EDHFs such as the 15-LO pathway function as an iEDHF while others are cEDHFs. The 15-LO pathway has many characteristics of an inducible pathway. The principle enzyme regulating the activity of the pathway is 15-LO. It is regulated in multiple ways including transcription, translation and epigenetics, and by many conditions and hormones such as hypoxia, anemia, hypercholesterolemia, atherosclerosis, estrogen, ILs and likely others (angiotensin, aldosterone, cytokines and growth factors). While elevated in newborn and young animals, the 15-LO pathway is suppressed and dormant in older animals but can be reactivated. Hypoxia, hypercholesterolemia, estrogen and IL-13 increase the expression of 15-LO, the synthesis of vasoactive 15-H-11,12-EETA and 11,12,15-THETA, increases membrane hyperpolarization to AA and increases relaxations to AA and acetylcholine. When activated, the 15-LO pathway is additive with the COX and NOS vasodilatory pathways and synergistic with a cEDHF. Additional studies are needed to fully define the regulation of 15-LO-iEDHF and its role in pathological and physiological settings. It is possible that EDHFs do not vary with the vascular bed and species but are suppressed in some vascular beds and species and induced in others. Understanding the mechanisms regulating the expression of various EDHFs may define which are cEDHFs and iEDHFs and clarify the vascular and species distribution of EDHFs and their relative activities.

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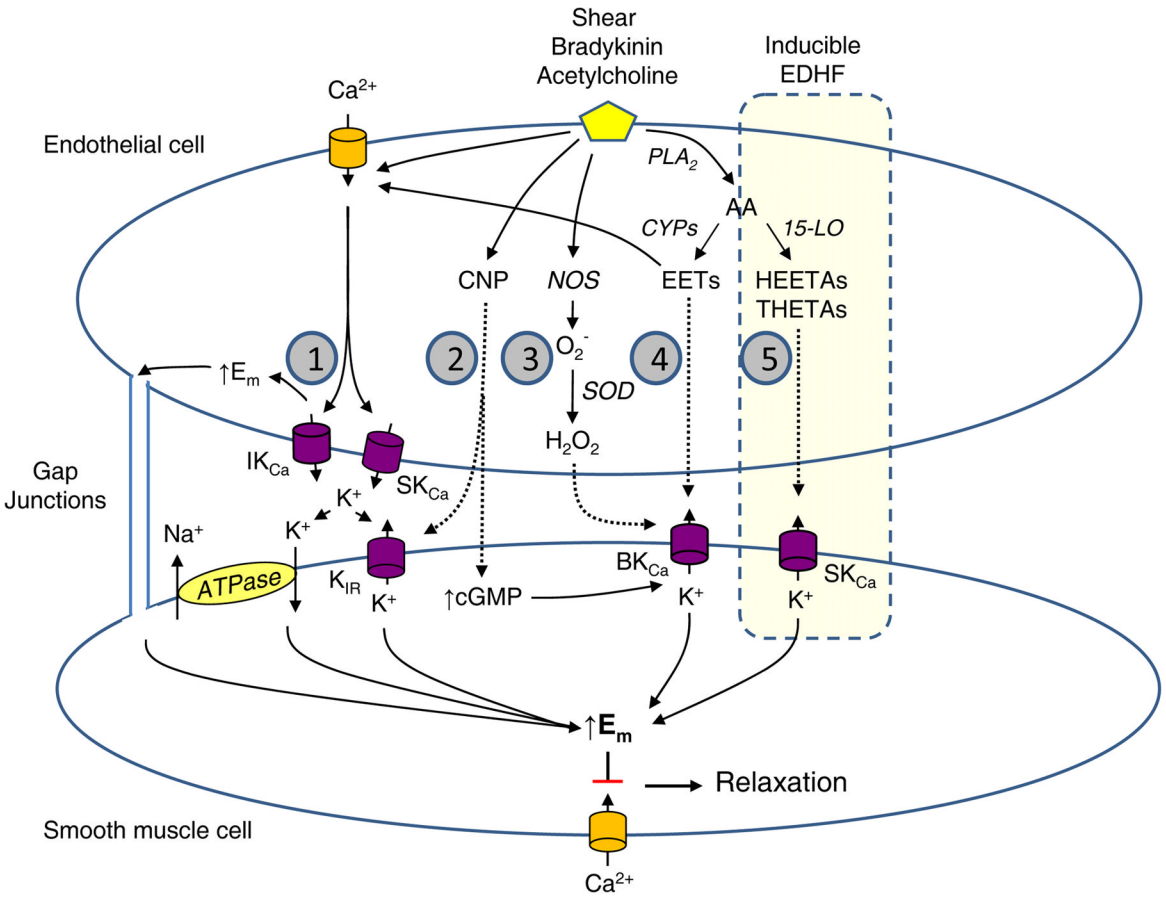

Figure 1.

Signaling mechanisms of the five major endothelium-derived hyperpolarizing factors (EDHFs). Shear stress or endothelium-dependent agonists including acetylcholine and bradykinin stimulate EDHF-dependent vascular relaxation. EDHF mediators include: (1) electrical transmission of endothelial hyperpolarization through myoendothelial gap junctions, (1) K ion, (2) C-type natriuretic peptide (CNP), (3) hydrogen peroxide (H_2O_2), (4) epoxyeicosatrienoic acids (EETs) and (5) 15-lipoxygenase-1 (15-LO-1) metabolites, 15-H-11,12-EETA and 11,12,15-THETA. The 15-LO-1 inducible EDHF pathway is highlighted. The shaded numbers in the figure refer to the Classification of EDHF section of the text and correspond to numbered descriptions of 5 major EDHFs.

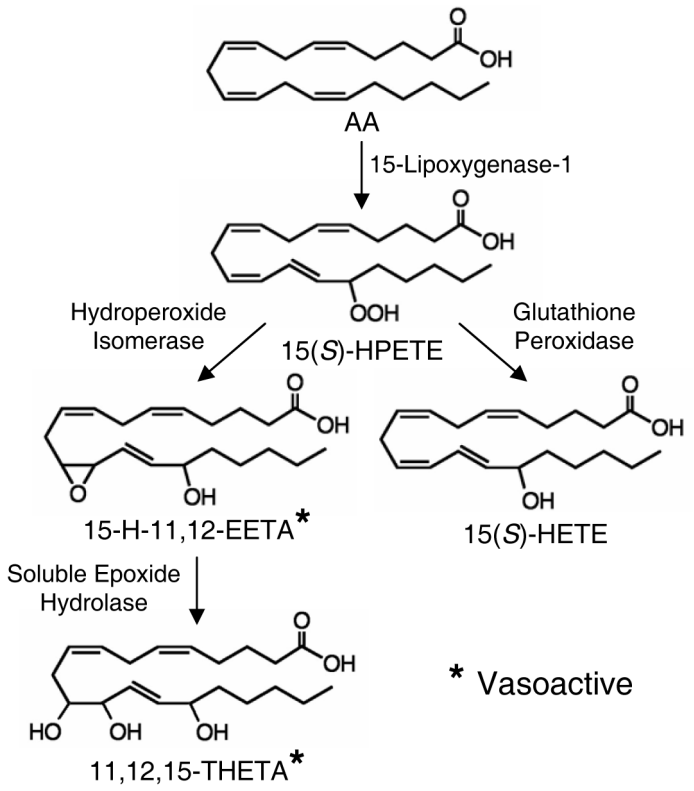

Figure 2.

15-Lipoxygenase-1 pathway of endothelial cell arachidonic acid (AA) metabolism. 15(S)-hydroperoxyeicosatetraenoic acid (15(S)-HPETE), 15-hydroxy-11,12-epoxyeicosatrienoic acid (15-H-11,12-EETA), 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), 11,12,15-trihydroxyeicosatrienoic acid (11,12,15-THETA). * 15-H-11,12-EETA and 11,12,15-THETA cause vascular relaxation and function as EDHFs.

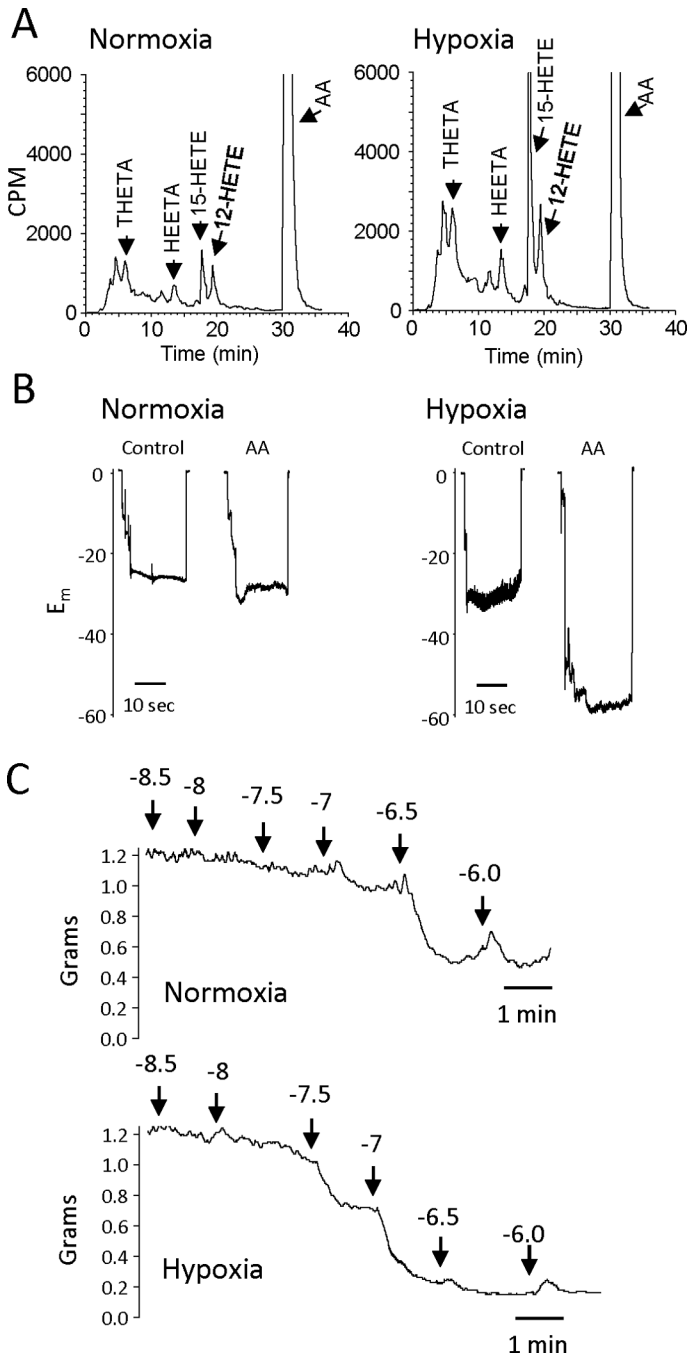

Figure 3.

Effect of hypoxia on 15-LO activity in the rabbit vasculature.

(A) AA metabolism of rabbit aortic endothelial cells exposed to normal oxygen (21% O_2, normoxia) or reduced oxygen (0.7% O_2, hypoxia). Endothelial cells were incubated with ^{14}C-AA in the presence of indomethacin (10 μM) for 8 h under normoxic or hypoxic conditions. The media was removed, extracted and metabolites resolved by reverse-phase HPLC. Radioactivity of column fractions was measured by scintillation counting. Migration times of known standards are noted on each chromatogram.

(B) AA-induced hyperpolarization responses in mesenteric arteries from normoxic or hypoxic rabbits. Male 8 week old rabbits were exposed to either normoxic conditions (21% O_2) or hypoxic conditions (12% O_2) for 5 days. Membrane potential cell impalement recordings were made in the freshly dissected arterial segments incubated with indomethacin (10 μM) and phenylephrine (100 nM) with or without AA (10 μM).
(C) Acetylcholine relaxations of mesenteric arteries from normoxic (top trace) or hypoxic (bottom trace) rabbits. Arterial rings were mounted in a myograph, stretched to a basal tension of 1 gram, treated with indomethacin (10 μM) and N-nitro-L-arginine (30 μM) and constricted with phenylephrine (0.1 – 1.0 μM). Increasing concentrations of acetylcholine were added and relaxation responses recorded.

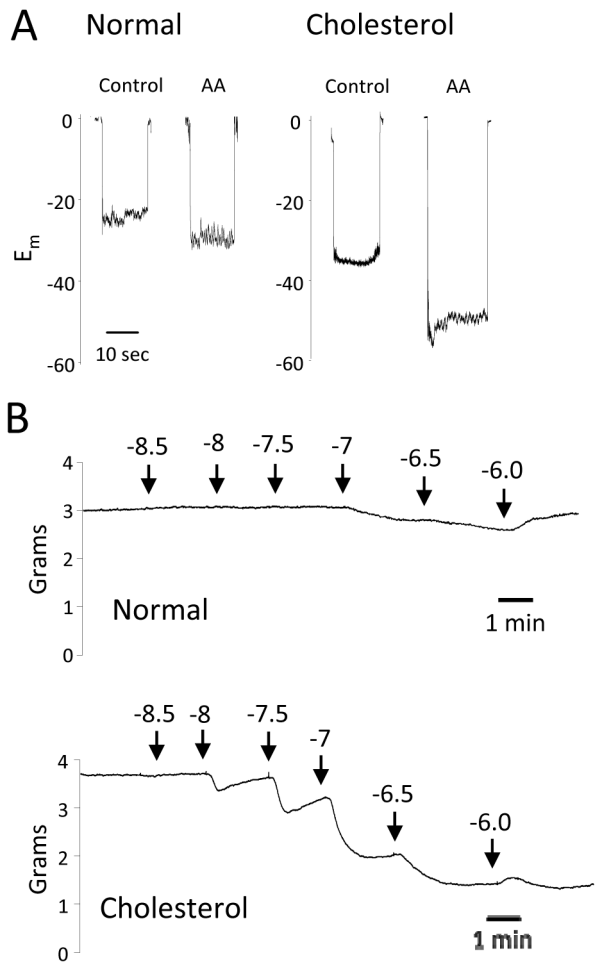

Figure 4.

Effect of a high cholesterol diet on aortic vascular activity. Male 8 week old rabbits were fed either normal chow (normal) or cholesterol enriched (2% cholesterol) chow (cholesterol) for 2 weeks.

(A) AA-induced hyperpolarization responses. Membrane potential cell impalement recordings were made in the freshly dissected aortic segments incubated with indomethacin (10 μM) and phenylephrine (100 nM) with or without AA (10 μM).

(B) Acetylcholine relaxations. Arterial rings from rabbits fed normal chow (top trace) or rabbits fed cholesterol-enriched chow (bottom trace) were mounted in a myograph, stretched to a basal tension of 2 grams, treated with indomethacin (10 μM) and N-nitro-L-arginine (30 μM) and constricted with phenylephrine (0.1 – 1.0 μM). Increasing concentrations of acetylcholine were added and relaxation responses recorded.

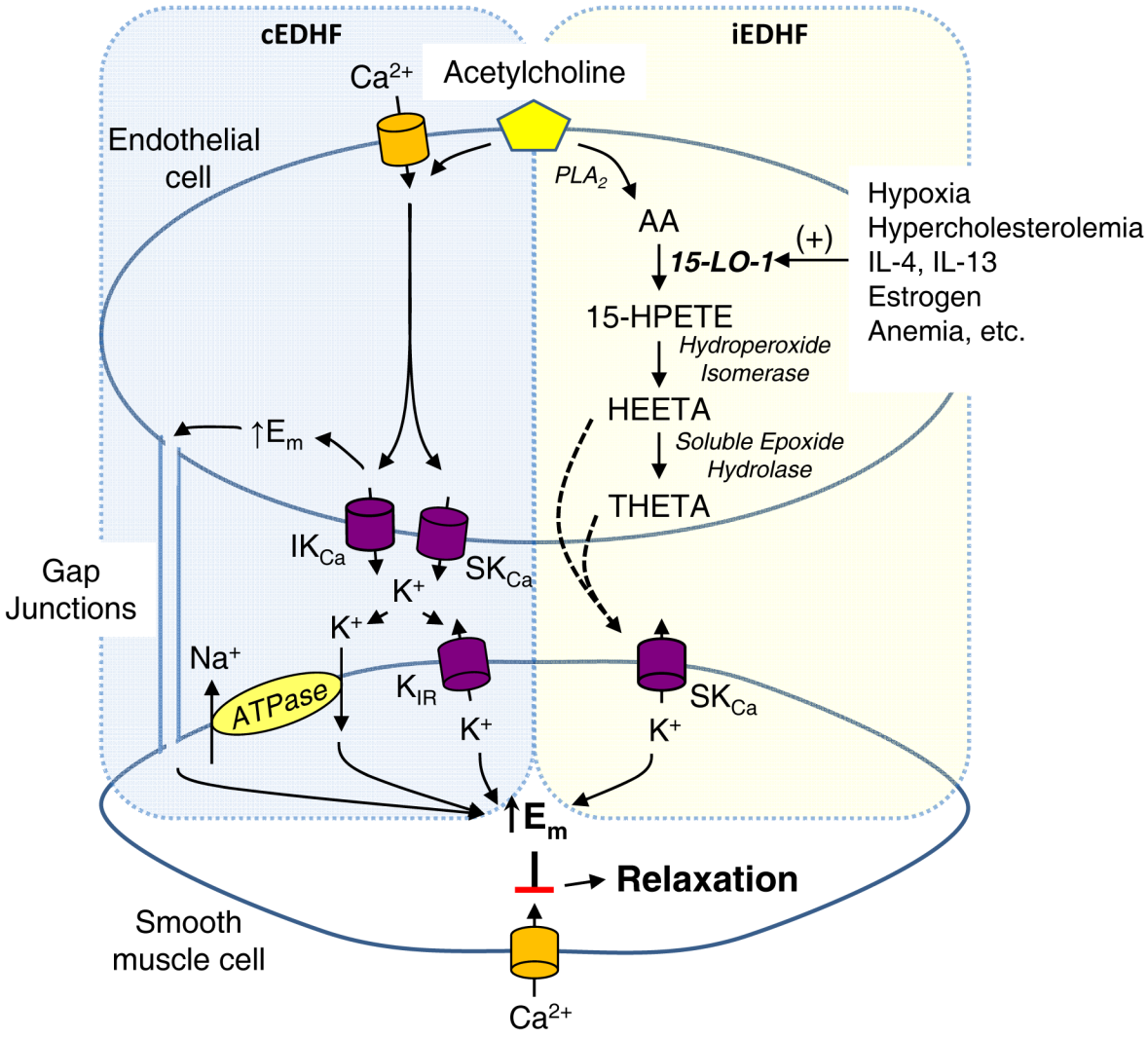

Figure 5.

Co-release of constitutive (c) and inducible (i) EDHFs mediate synergistic vasorelaxation. In vascular endothelial cells, acetylcholine activates; 1) calcium influx which stimulates IKCa and SK_{Ca} channels resulting in K ion efflux (the cEDHF pathway) and 2) PLA2 release of AA from membrane phospholipids. AA is metabolized by 15-LO-1 to HEETA and THETA (the iEDHF pathway). 15-LO-1 expression is increased by hypoxia, hypercholesterolemia, interleukin-4 (IL-4), interleukin-13 (IL-13), estrogen and anemia. The cEDHF and iEDHF pathways cause smooth muscle hyperpolarization via distinct synergistic mechanisms. For the cEDHF pathway, endothelial cell hyperpolarization from K ion efflux is transmitted to the smooth muscle through myoendothelial gap junctions or K ions activate smooth muscle K_{IR} channels and the Na/K ATPase. HEETAs and THEETAs from the iEDHF pathway activate smooth muscle SK_{Ca} channels.