The role of endothelin in stroke: Experimental data and underlying pathophysiology

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Abstract

The role of endothelin in functional and structural changes after stroke is only partly understood: synthesis and release of the potent vasoconstrictor peptide endothelin-1 increases following ischemia and mediates the delayed hypoperfusion associated with ischemia, but the role of the other two endothelin subtypes remains unclear. Only little is known about its mode of action in ischemia. Recently developed selective peptide and non-peptide antagonists and agonists acting at the level of endothelin receptors provide useful tools for further investigation of the physiological role of endothelin and its impact on pathophysiological processes in different neurological diseases including ischemic, nonhemorrhagic stroke and cerebral vasospasm. The present review supports experimental and clinical evidence that vasoactive mediators, and endothelin-1 in particular, are important contributors to the pathogenesis of cerebral ischemic damage in animal models and in humans. Special reference is given to explain the few clinical data in the light of experimental results.

Key words: cerebral ischemia, endothelium, endothelin, cerebral blood flow.

Introduction

In 1988, endothelin (ET) was discovered, that is currently known as one of the most potent endogenous vasoconstrictors [1]. Three isopeptides (ET-1, ET-2, ET-3) have been described and were implicated in different cerebrovascular pathologies, in part due to their potent vasoconstrictor capacity [2]; ET-4 is restricted to cells outside the central nervous system (CNS) [3, 4]. Physiological effects of these four ET subtypes are mediated via two receptor subtypes, endothelin type A (ET_A) and endothelin type B (ET_B). Besides endothelial cells, potential sources of ET in the CNS include vascular smooth muscle [5], neurons [6] and glia [7]. The predominant physiological impact of ET_A activity harbors vasoconstriction, whereas ET_B receptor – mainly seen on endothelial cells – appears to mediate vasodilatation [8].

Recently, the knowledge about physiologic effects of ET in the CNS has accumulated rapidly, but only little is known about its mode of action during ischemia [9]. The aim of this review was to summarize the physiological role of ETs or ET receptors in cerebral arteries and the changes induced by its regional up-regulation after ischemic stroke.

Methodology

Medline and Embase databases were searched for trials, case series, or case-reports published between 1966 and August 2003 or 1986 and

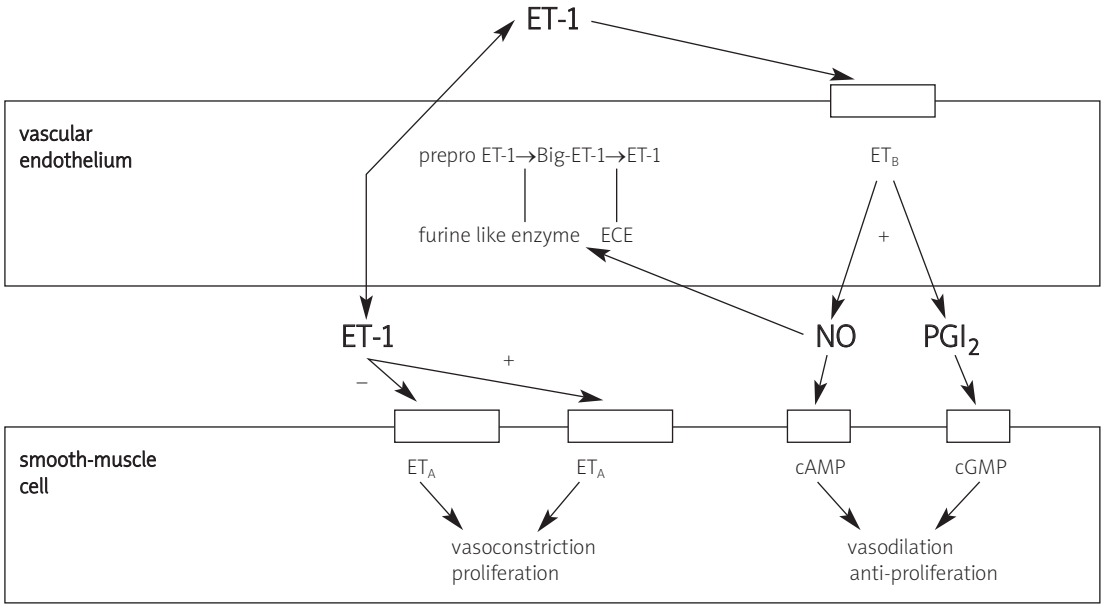
August 2003, respectively. The terms used included *endothelin, endothelin-1, receptor, stroke, cerebral ischemia, cerebral infarction, middle cerebral artery, hemorrhage or cerebral vasospasm*. The manuscript had to be published or translated in English, French or German. More recent publications were used in preference to older ones, as the purpose of the review is to give an up-to-date review of the current experimental and clinical knowledge of the role of ET in stroke. Older publications were retrieved if they were cited in the selected publications and deemed significant for the purposes of this review.

Biochemistry and molecular biology of endothelin

Synthetic pathway and breakdown

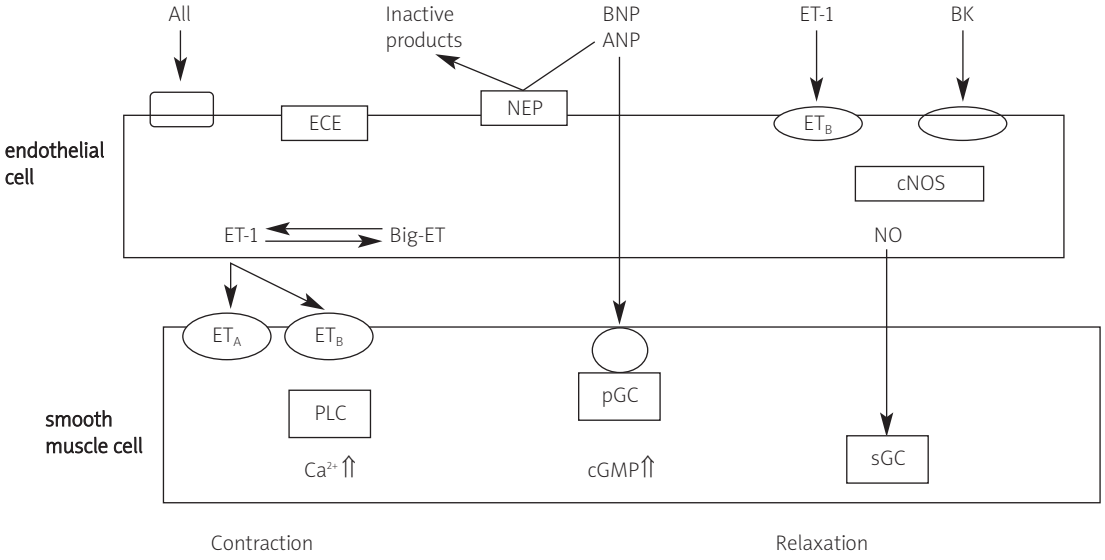
The four distinct ET isopeptides (molecular mass of 2492; composed of 21 amino acid residues with free amino/carboxy termini) are produced intracellularly as large prepro-ET (approximately 200 amino acids) and are cleaved at two sites by a neuronal endopeptidase, furin, forming biologically inactive precursor "big-ET" (Figure 1) [1]. Subsequent hydrolysis to final biologically active peptide, ET, and inactive C-terminal fragment is catalyzed by ET-converting enzyme (ECE) [10, 11]. In the CNS, this proteolytic process occurs at different bonds (Trp21-Val22 for ET-1 and ET-3; Trp-lic for ET-2) with distinct ECEs (17). ECE represents a zinc metalloprotease that is associated with a strict substrate specificity [12]: ECE-1 is a membrane-bound ectoenzyme (endothelial cells) [13, 14], whereas ECE-2 is localized intracellularly (vascular smooth muscle cells) [13]. ECE-1 isoform differs in its regional membrane distribution, but its affinity toward big-ET-1 remains comparable [14].

ET causes vasodilatation at lower concentrations and marked or sustained contraction at high concentrations [15]. The dilator or constrictor response evolves activation of ET receptors (ET_A and ET_B receptors) linked to NO and/or prostacycline release by endothelial cells [16]. In addition, ET-1 has mitogenic and proliferative properties [17] and acts in paracrine fashion initiating endothelial secretion of vasoactive factors that contribute to local CBF regulation [18]. Effects of ET on neurotransmission acting as neuropeptides [19] and on neuronal excitability [20] are also described. An important mechanism in regulating actin organization of astrocytes has also been suggested reflecting functional astrocytic changes seen following ischemic damage [21]. A rapid increase of intracellular calcium concentration ($[Ca^{2+}]_i$) through ET_A-mediated activation of phospholipase C (PLC) or G-protein is responsible for many actions of ET [22] and contains two components: an instant Ca^{2+} spike seconds after the application of ET (increase in $[Ca^{2+}]_i$ due to release from intracellular store), and then a sustained $[Ca^{2+}]_i$ elevation that lasts for several minutes and depends on the influx of $[Ca^{2+}]_e$ [23]. ET-1-induced sustained increase in $[Ca^{2+}]_i$ is the result of Ca^{2+} influx through voltage-independent Ca^{2+} channels (VICCs) in addition to voltage-operated Ca^{2+} channels (VOCCs) [24]. These VICCs (associated with ET_A) consist of two types of Ca^{2+}-permeable nonselective cation channels (NSCC-1 and -2) and one store-operated Ca^{2+} channel (SOCC) [25].



Legend: ET-1: endothelin-1; ECE: endothelin converting enzyme; ET_A: endothelin A receptor, ET_B: endothelin B receptor; PGI_2: prostacyclin

Figure 1. Biology of endothelin



Legend: ET-1: endothelin-1; ECE: endothelin converting enzyme; ET_A: endothelin A receptor; ET_B: endothelin B receptor, PLC: phospholipase C, ANP: atrial natriuretic peptide; BNP: brain natriuretic peptide; NEP: neutral endopeptidase; pGC: particulate guanylate cyclase; sGC: soluble guanylate cyclase; cGMP: cyclic guanosin monophosphate; cNOS: constitute nitric oxide synthase, NO: nitric oxide; BK bradykinin

Figure 2. Regulation, processing and secretion of proteins related to endothelin-1

ET receptors

ET act through a number of specific G-protein linked receptors of which several have been cloned so far (Figures 2 and 3) [25]. Three subtypes of mammalian ET-receptors have been identified: ET_A (50-70 kDa), ET_{B1} and ETB2 (30-40 kDa). The amino acid structure of both subtypes is in 50% identical, and each subtype can be frequently found across mammalian species (85-90%) [26]. ET-1 induced constriction occurs via ET_A in the smaller resistance vessels [26]. However, ET_B can act either as vasoconstrictor or as vasodilator depending on the species or the specific type and location of the vasculature [27]. ET_A and ET_B are up-regulated in ischemic stroke [28].

ET_A is found predominantly in cerebrovascular smooth-muscle cells, mediates a potent and long-lasting vasoconstriction or cell proliferation and preferentially binds ET-1 (Table I) [29]. ET_A-induced vasoconstriction is related to the receptor's ability to stimulate PLC, which leads to the formation of inositol 1,4,5-tripshosphate and diacylglycerol (Figure 2) [25]. The former increases $[Ca^{2+}]_i$, which in turn causes increased myosin light chain phosphorylation and vascular smooth muscle cells contraction [30]. This vasoconstriction persists after ET-1 is removed from the receptor [31], probably because the $[Ca^{2+}]_i$ remains elevated. NO shortens the duration of vasoconstriction by accelerated return of $[Ca^{2+}]_i$ to its basal concentration [32]. Diacylglycerol and calcium stimulates proteinkinase C (PKC), which mediates the mitogenic action of ET-1 [33]. ET_B is distributed generally on vascular endothelial cells and follows the endothelium-dependent vasodilatation via NO and prostacyclin-synthesis [34]. This effect is equal for ET-1 and ET-3. Activation of the ET_{B2} on vascular smooth muscle cells causes vascular constriction in peripheral vessels [34], but so far, this has not been proved for cerebral circulation. Recent data, however, suggest an ET_B-dependent delayed vasoconstriction in subarachnoid hemorrhage (SAH) [35]. The (intra)cellular effect of ET_B activation is similar to that of ET_A activation in stimulating PLC activation, the generation of inositol 1,4,5-triphosphate or diacylglycerol, and the different Ca^{2+}-mobilization mechanisms, including Ca^{2+} release from intracellular Ca^{2+} stores or activation of L-type and nonselective Ca^{2+} channels [25]. However, ET_B is linked to inhibitory G proteins, which in some cells leads to inhibition of cAMP generation and activation of Na^+-H^+ antiporter [36]. Binding of ET-3 to ET_B causes transient vasodilatation and is probably caused by increased NO and prostcyclin production [37].

ET and intracellular transduction mechanism

ET-induced vascular smooth muscle contraction develops or relaxes slowly and is dependent on $[Ca^{2+}]_e$ [27, 38]. It has been hypothesized that Ca^{2+}-independent isoforms of PKC are activated by ET and thereby produce sustained, irreversible vasoconstriction. The cloning of the Ca^{2+}-regulated cytoplasmic proline-rich tyrosine kinase (PYK2) suggests a link between G protein-coupled receptors and induction of tyrosine phosphorylation via mobilization $[Ca^{2+}]_i$ [39]. Moreover, PYK2 plays an important role in coupling

Table I. Biological characteristics of endothelin

• ET consists of three distinct 21-amino acid peptides: ET-1, ET-2 and ET-3
• ET has two main receptors: ET_A (affinity ET-1>ET-2) and ET_B (ET-1=ET-2=ET-3)
• Synthesized in blood vessels, brain, and other mammalian cells
• Produces prolonged and profound vasoconstriction at low doses
• Hemolysate, hemoglobin, thrombin, angiotension II, vasopression, calcium, and TNF-α can cause increased production and release of ET from endothelium
• Increased concentration are found in the cerebrospinal fluid postischemically
• Activation of ET_A and ET_B can stimulate increased production of ET-1 (vasoconstrictor) and NO (vasodilator)
• ET-1 causes calcium influx, intracellular calcium mobilization, activation of proteinkinase C and phospholipase A2 and C, Na^+/H^+ exchange, and stimulates Na^+/K^+-ATPase, calcium stimulated K^+ channels or $Na^+/K^+/Cl^-$ co-transport

Legend: ET, endothelin; ETA, endothelin receptor type A; ETB, endothelin receptor type B; TNF, tumor necrosis factor

G protein-coupled receptors with extracellular signal-regulated kinase activation [40].

ET-3 probably acts through ET_B to stimulate NO production by endothelial cells and may be part of paracrine/autocrine system of regulation of these vasoactive substances. Both ET-1 and ET-3 stimulate the production of several prostaglandins thereby contributing to the inhibition of platelet aggregation and transient vasodilatation, both by means of prostacyclin [41].

Ion channels

ET-1 inhibits the activity of K^+_{ATP} channels [42] and increases Cl_{Ca} currents in smooth muscle cells of cerebral arteries [43]. Despite the initial (intra)cellular similarities of astrocyte and cerebrovascular smooth muscle responses, differences appear during prolonged exposure to ET-1 [44]. Sustained exposure to ET-1 is associated with a loss of cell-to-cell coupling within the astrocyte-containing microvasculature leading to decreased ionic currents from astrocytes [44] and to a dysregulation of the multicellular functional units that may prevent control of capillary blood flow [45]. Consistent with this possibility, it has been demonstrated that astrocytic uncoupling, which occurred between 5-15 minutes after exposure to ET-1, is temporally associated with reduced astrocytic conductance [44]. In addition, ET-1-induced inhibition of gap junction permeability can be antagonized by ATP-sensitive K^+ channel blockers [46], pointing out that this mechanism may play a role in ischemic stroke [47].

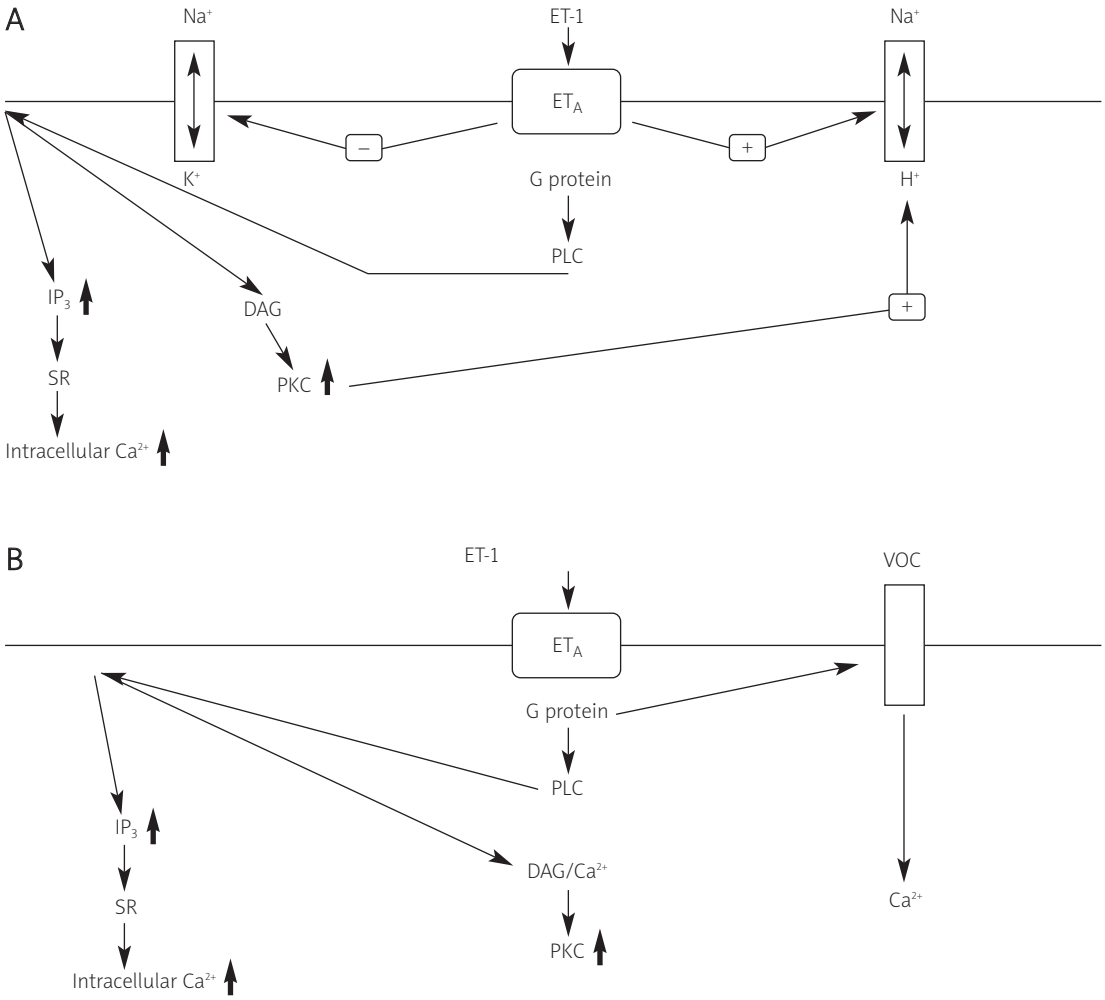
In addition to VOCC, ET-1 activates VICCS such as NSCC-1 or NSCC-2 and SOCC in cerebrovascular smooth muscle cells [48]. $[Ca^{2+}]_e$ influx through NSCC-1, NSCC-2, and SOCC plays an essential role for ET-1-induced PYK2 phosphorylation in cerebrovascular smooth muscle cells [49]. In addition, phosphoinositide 3-kinase seems to be involved in ET-1-induced PYK2 phosphorylation that depends on the $[Ca^{2+}]_e$ influx through SOCC and NSCC-2 [50]. ET-1 and ET-3 are the only peptides known to activate the Na^{2+}-K^+-Cl^- transporter function of brain capillary endothelial cells leading to the maintenance of a low-potassium environment [51], which is important in neuronal depolarization and the subsequent conduction of impulses between neurons.

Regulation and expression of endothelin receptors

The regulation of ET receptor expression often parallels that of ET. For instance, cerebral ischemia rapidly stimulates the production of ET-1 and ET_A receptors in endothelial cells and vascular smooth-muscle cell, respectively [33]. This induction of peptinergic activity is followed of increased local perfusion. Epidermal growth factor, basis fibroblast growth factor, cyclic AMP, or estrogen up-regulate ET_A in some tissues, selective up-regulation of ET_B is seen after focal cerebral ischemia. In addition, C-type natriuretic hormone, angiotensin II, perhaps basic fibroblastic growth factor, and transforming growth factor B down-regulates ET_A, whereas cyclic AMP and catecholamines down-regulate ET_B [26]. Differences in tissue-specific expression of the two main receptor subtypes contribute to specific actions of the ETs.

Role of endothelin in stroke

Mechanisms contributing to evolution of ischemic brain damage are complex, multifactorial [52] and are only partly understood. ET-1 has been demonstrated to override cerebral autoregulatory mechanisms, to constrict a number of cerebral vessels in vivo and to reduce CBF below the ischemic threshold to induce infarction [53]. However, direct neurotoxic effects of ETs are discussed controversial, but it seems that possible brain damage is more likely a result of the potent vasoconstrictor activity of ET than a direct neuronal effect [54, 55].



Legend: ET-1: endothelin-1; ETA: endothelin A receptor; ETB: endothelin B receptor; PLC: phospholipase C, DAG; Diacyl-glycerol; IP3: Inositol-tris-phosphate; SR: sarcoplasmic reticulum; VOC: voltage operated calcium channel

Figure 3. The mechanism of signaling induced by endothelin-1 through the endothelin-A transmembrane receptor
A – endothelin receptor A; B – endothelin receptor B

Pathophysiological alterations of cerebral blood vessels during ischemia

1. Endothelin

As ischemic brain damage is initiated by a series of pathophysiological events occurring at the blood-vascular-parenchymal interface, endothelial dysfunction may be due to an imbalance between NO and ET-1 production underlined by the fact that ET-1-levels in brain tissue (up to 4-fold) and brain extracellular fluid (up to 10-fold) are increased following ischemia compared to their normal plasma concentrations [56]. These elevated ET levels are damage-dependent and are due to an increased synthesis in neuronal and endothelial cells, delayed synthesis in reactive astrocytes, infiltrating leukocytes or activated microglia and macrophages [57]. ET-1 gene expression is enhanced by several ischemia-related factors including thrombin, transforming growth factor-β, hemoglobin, and TNF-α [58] and is inhibited by NO and cGMP. A common sequence for acute-phase reactant elements is present in the promoter of the ET-1 gene, but in contrast to the gene for endothelial NO synthetase, activation of this element decreases transcription of the ET-1 gene [58]. mRNA levels reach its maximum at 24 hours after ischemia, while the contractile response demonstrates its greatest intensity at 48 hours. During development of infarction, neuronal ischemic damage induces proliferation of microglia and astrocyte [59]. As glial cells synthesize ET during active gliosis [60], reactive astroglia may represent another good candidate for ET-1 production. In addition to vasoconstrictor activity,

it has been demonstrated that ET-1 also promotes leukocyte adhesion [61], thrombus formation [62], BBB permeability (probably by affecting endothelial permeability) [63], brain edema formation [64] or neuronal modulation [59], all of which could adversely influence microvascular perfusion. However, the physiological balance between ET-1 and NO can be disturbed during ischemia by further increasing physiological inhibition of ET-1 production and release from endothelial cells by NO [65]. Indeed, ET-1 concentrations at the interface of the endothelium and smooth muscle cells are higher than those in the bloodstream.

ET-1 is mainly involved in the pathogenesis of ischemic brain damage. The delayed onset of postischemic hypoperfusion after transient global ischemia is associated with increased ET-1 levels in CSF, and can be reversed by pharmacological treatment with selective ET_A antagonists [66]. Furthermore, ET_A antagonists also attenuate neuronal loss demonstrating a direct link between preservation of perfusion and reduction of ischemic brain damage. However, data regarding ET-1 plasma levels in acute ischemia remain controversial, as several studies have reported normal ET-1 levels during various phases of ischemia [65-67]. These variable ET-1 levels could be attributed to its in vitro/in vivo actions, regional differences within the brain [68], pharmacological dosage used [69], receptor subpopulation and their density [70], or relevant vasodilator substances [71]. Additionally, these conflicting effects of ET during ischemia may be due to the different timing of sampling in the various studies [72]. However, ET plays an important role in the physiological and pathophysiological regulation of the smooth muscle contraction in the cerebrovascular system: ET-1 levels are increased within 24 hours after the onset of ischemic symptoms, whereas they are normal if the sampling is performed after 24 hours. It has been speculated that increased ET-1 levels may be due to over-production of ET-1 or leakage of ET-1 from damaged endothelium of microvessels involved in cerebral infarction [53]. ET-1 is released to the abluminal vessel side [73, 74], so that plasma levels might not reflect local production of ET-1. This is consistent with the hypothesis that ET-1 is a local regulating factor and explains the findings, that an individual response to different pathophysiological stimuli to stress in addition to the acute phase of ischemia may also account for controversial data obtained in the above-cited studies, and for fluctuation of ET-1 secretion.

ET may cause a redistribution of the blood flow and oxygen consumption within the ischemic region: oxygen supply/consumption balance is altered by experimental application of ET-1 in both normal and ischemic brain regions [75]. Similarly, if ET-1 causes some vasoconstriction in the border region, this action could also redistribute blood from the non-ischemic to the ischemic region, thereby influencing the microvascular O_2-supply/-consumption balances. Intravenous infusions of ET-1 to healthy volunteers decrease the arterial-venous oxygen differences across the cerebral vascular beds, suggesting some form of vasodilatation [76]. Despite the lack of significant changes in local blood flow or O_2-consumption, a significant improvement in the O_2-supply/-consumption-ratio has been found after ET-1 treatment in ischemic cortex [77]. The venous oxygen saturation of the ischemic cortex is significantly lower than the contralateral cortex both in the control and ET-1-treated groups. ET treatment also decreased the number of small veins with low oxygen saturation in the ischemic cortex [77]. These oxygen balance effects appear to be local, since local application of ET-1 did not affect global hemodynamics. ET reduced the wide variability in venous O_2-saturation found in the ischemic cortex as determined by a reduction in the coefficient of variation of venous O_2-saturation.

The amount of plasma ET-1 levels correlates with infarct volume and a pathophysiological role of the peptide in outcome after ischemic stroke has been hypothesized [53]. There are, however, several intrinsic mechanisms, which may limit these effects of ET-1. First, the prepro-ET-1-mRNA is particularly short lived and this may be a safeguard against overproduction. Moreover, vasoconstrictive effect of ET is contrasted by the concomitant release of NO, prostacyclin and atrial natriuretic peptides (ANP). In vitro studies suggest that ET-1 stimulates NO release via activation of ET_B on vascular endothelium. Recently, the plasma ANP as well as ET-1 levels measured in patients with acute stroke on admission, and 3 and 7 days thereafter were reported to be significantly higher than in controls, suggesting that such high ANP levels might reflect a vasodilatory response to limit the effect of ET-1 [78]. Indeed, ET-1 stimulates ANP release in vitro and in vivo, counteracting the potent action of ET-1 [79]. Furthermore, ANP acts directly on damaged brain to inhibit water and sodium accumulation in ischemic brain edema. Therefore, ANP could act as a protective factor in ischemic stroke via both vasodilator and antiedema effects. At the present time, however, it is not possible to draw any conclusion from these experimental or clinical data.

2. Endothelin-receptor

Cerebral ischemia seems to have an impact on the intracellular pathway as described by transcription of both ET-receptors in vascular smooth

muscle cells in the affected hemisphere [34]. ETA up-regulation is mediated via increased transcription and subsequent translation of ETA mRNA [80]. The mRNA level reaches its maximum after 24 hours of beginning of ischemic stroke, while the contractile response has its maximum at 48 hours [34]. The 5´flaning region of the genes encoding the ET-receptors contains several regulatory elements, such as GATA motifs and E boxes [81]. One possible reason of regional up-regulation of ET-receptors might be a coupling to the induced changes in intracellular pathways that occur during and after cerebral artery occlusion [28].

The neuronal expression of ET_A following cerebral ischemia suggests that ET may be involved in the pathology of stroke as ET_A are functionally linked to the influx of $[Ca^{2+}]_e$ and to the mobilization of $[Ca^{2+}]_i$ in neural cells [23]. The ET-stimulated increase in $[Ca^{2+}]_i$ in neurons may contribute to Ca^{2+}-overload observed in ischemia [82]. An excess $[Ca^{2+}]_i$ seems to be a major etiological factor of neuronal death [83] as the high $[Ca^{2+}]_c$ may activate a variety of Ca^{2+}-stimulated enzymes including proteases, lipases, nucleases, protein kinases and inducible nitric oxide synthase (iNOS), which subsequently evoke the biochemical cascade that results in neuronal death [84].

In cultured mouse glial cells, ET_B appear to be linked to Ca^{2+}-signaling pathways [80, 85]. Furthermore, activation of ET_B in cultured rat glial cells led to iNOS expression inhibition induced by TNF-α, IL-1β or lipopolysaccharide (LPS) [86]. However, it has been reported more recently that activation of ET_B decreased IL-1β-induced release of NO but enhanced LPS-induced NO release from cultured rat astrocytes [85]. Although somewhat contradictory, these findings implicate an immunomodulatory role of glial ET_B. It is thus noteworthy that ET_B is expressed specifically in activated microglia after focal infarction [59]. As ET-1- and ET-3-like immunoreactives were enhanced within astrocytes in damaged neuronal tissue [87], it should also be noted that astrocytic expression of both ET_A and ET_B has not been observed [59].

Effect of endothelin-1 on BBB permeability in cerebral ischemia

During focal cerebral ischemia, capillary perfusion may either not be altered [88] or decreased [89]. Since ET-1 is a strong vasoconstrictor, ET-1 may affect capillary perfusion and perfused capillary surface area causing alterations of transfer coefficient across the blood brain barrier (BBB) [90]. Direct administration of ET-1 to the brain surface after induced focal ischemia demonstrated no significant alteration of the transfer coefficient of ^{14}C-aminoisobutric acid at 1-2 hours after application [91], suggesting an unchanged BBB permeability. But at 48 hours following intrastriatal injection of ET-1, a significant increase in brain penetration of a fluorescent tracer (lucifer yellow) is reported [92]. Intrathecal administration of ET-1 results in prolonged breakdown of the blood-spinal cord barrier [93]. For this reason, it can be hypothesized that ET-1 may cause delayed BBB disruption.

Application of ET-1 to the ischemic brain tissue attenuates an increased transfer-coefficient [93], but it cannot be determined whether the decrease in transfer coefficient is due to decreased perfused capillary surface area or permeability or both, the underlying pathophysiological mechanism remains unclear. It is possible that ET-1 causes vasoconstriction and thus decreases the perfused capillary surface area. However, CBF in ischemic brain region is not altered by ET-1 application. Therefore, it is difficult to accept vasoconstriction as the primary mechanism for decreased transfer coefficient by ET. As NO also has been reported to affect BBB function [94], it is also possible that increased BBB permeability in ischemic area is caused by NO, and that antagonistic effect of ET-1 on BBB reverse the increased permeability. However, stimulation of ET receptors activates NO synthase (NOS), so that the final status of BBB disruption in ischemia may be the net effect of ET-1, NO, and other substances which may affect BBB function and the status of cerebral metabolism.

Endothelin-induced inhibition of gap junction permeability

Ischemic stroke is followed by increased tissue ET-1 levels and hypertrophic-appearing reactive astrocytes [95], suggesting a role of ET-1 in the activation of astrocytes [96]. In damaged brain, astrocyte hypertrophy and increased GFAP immunoreactivity have been described upon ET-1 application [97]. In addition, a reduction of gap junctional coupling is not only observed upon ET-1 treatment in vitro [98], but also in reactive astrocytes in vivo [99], suggesting that decreased astrocytic gap junctional permeability might play a key-role in ET-1-induced astrocytic activation (i.e. increased protein content and shape change) [54]. As tolbutamide also antagonized ET-1-induced morphological changes, an involvement of astrocytic gap junction permeability in reorganization of structural proteins induced by ET-1 can be demonstrated [47]. These data are in line with recent findings of altered cytoskeletal organization upon gap junction inhibition by octanol and 18α-glycyrrhetinic acid in dibutyryl-cAMP differentiated astrocyte cultures [100].

Astrocytic gap junctions play a role in propagation and amplification of cell damage [101]. Reduction of connexin 43 expression by antisense nucleotides decreased neuronal death in vitro [102]. In addition, pharmacological blockade of gap junctions effectively

reduces infarct size [103] and ET-1 protects astrocytes from ischemic brain damage [104]. Accordingly, the absence of functional ET_B led to an augmented ischemic brain damage [26]. These protective effects of ET-1 might also involve modulation of astrocytic gap junctional network.

Neuronal injury caused by endothelin in stroke

Astrocytes are proliferated or differentiated by ETs [105] and microglia may also be stimulated by ETs, since alveolar macrophages are activated by ET-1 to produce superoxide anions. Both astrocytes and microglia aggregated in the neuronal lesions can express both ET and NOS [26]. In the endothelium, stimulation of ET_B increases NO production [37], or ET and NO reciprocally modulate production and action [106]. In mesengial cells, ET-1 inhibits cytokine-stimulated transcription of iNOS, and in CHO cells expressing ET_A, NO terminates the ET-1 response [32]. Thus, it may be suggested that in the rat's brain, glial ET system and NOS may participate in neuronal damage, possibly through mutual regulation in astrocytes and microglia.

After ischemic stroke, increased ETB expression on astrocytes may be protective as a clearance mechanism to remove increased levels of peptide [107]; however, there is some evidence to suggest that ET's action on reactive glial cells may contribute to the neurotoxicity frequently observed following brain damage. Studies of Sasaki et al. [108] determined that stimulation of cultured astrocytes with ET-1 induces glutamate efflux via reversal of the Na^+ and/or K^+ glutamate transporters. Such down-regulation of the glutamate transporter GLAST in camp differentiated astrocytes stimulated with ET-1 suggests that ET stimulation of ET_B expressing astrocytes may contribute to elevating extracellular glutamate levels [109]. Additionally, in a rat model of transient forebrain ischemia, NOS activity levels are correlated with increased expression of ET_B on astrocytes and microglia up to 28 days after ischemia [109]. Thus, ETs acting through ET_B on astrocytes have been suggested to contribute to the enhanced NO release and delayed neuronal death [110]. Recent evidence indicates that antagonism of ET receptors may reduce reactive gliosis and thus promote neuronal regeneration and survival [97], even after ischemic stroke.

Astrocytes with intense ET-like immunoreactivity and microglia with rich expression of ET_B aggregate in hippocampus CA1 subfields show delayed neuronal death after transient forebrain ischemia in rats [111]. These data and the finding, that ET-1 does not exert neuroprotection, point against primary damage in ET_B deficiency during ischemia [105]. An induction of vasoconstriction of cerebral vessels by ET-1 results in neuronal damage [112]. Since ET-1 induced contraction is further augmented by ET_B blockade [113] or in ET_B deficiency, a shift of cerebral vessel tone toward vasoconstriction might well contribute to increased neuronal damage after ischemia [105]. Interestingly, expression of ET_B immunoreactivity is increased in reactive astrocytes of damaged brain indicating an attempt of damaged brain to restrict propagation of injury [105]. Finally, microglial ET_B signaling also modulates brain damage [112].

Clinical aspects of endothelin in stroke

ET-1 levels are significantly higher in CSF of patients with large cortical than with smaller subcortical infarctions [65]. Higher plasma ET-1 levels in patients with cardioembolic stroke may be explained by warfarin use [114].

Different risk factors for stroke are associated with increased ET levels. One study demonstrates a correlation between plasma ET activity and age [74], whereas several others do not show any difference [115, 116]. A significant positive correlation is reported between cholesterol and ET-1 levels [116]. The elevation of plasma ET-1 activity observed in hypercholesterolemic patients even without atherosclerotic lesions may reflect endothelial dysfunction [117]. Arterial hypertension correlates with ET-1 levels [72], although perfectly normal ET-1 levels were also observed [116]. These data may contribute to enhancement of severity and size of infracted tissue by the amount of increased ET-1 levels in stroke patients.

Neuroprotective effect of endothelin receptor antagonist in stroke

ET receptor antagonists have been shown to reduce brain damage after focal cerebral ischemia [9, 118]. A critical evaluation of these data is difficult because there exist differences in experimental design, species, and receptor antagonists used. Most of the studies that have intervened with ET receptors antagonists after focal ischemia have focused on the role of ET_A as mediators of brain damage. The role of ET_B in ischemic disease, however, is still to be elucidated.

The development of specific ET receptor antagonists began in the early 1990s. Since that time, selective ET_A or mixed ET_A/ET_B antagonists have been shown in some, but not all, studies to increase CBF or improve neurological outcome and to reduce infarction volume in different models of global and focal cerebral ischemia [119]. One of the key issues is whether selective ET_A or mixed ET_A/ET_B antagonists are likely to be effective in treating ischemic stroke [120]. Recently, it was reported that selective ET_A antagonism provides protection after

focal ischemia, whereas the role of mixed ET_A/ET_B antagonists is not yet clarified [121]. In addition, it has been suggested that ET_B may be a clearance receptor in ischemic stroke. The direct adventitial application of an ET_B agonist onto dilated arterioles after focal ischemia showed that ET_B receptor-induced dilation in normal pial arterioles is lost in postischemic vessels suggesting that ET_B receptor-induced vascular effects may vary in pathologic situations such as acute stroke. More importantly, ET_{B1}/ET_{B2} antagonist in dilated arterioles, compared to vehicle treated control group, suggest that ET_B participate significantly in restricting postischemic dilatation of cortical arterioles in the ischemic penumbra [118]. Nonetheless, this contribution of ET_B in limiting vasodilatation is less pronounced compared with that of ET_A [122]. ET_A blockage completely prevented the development of brain edema by decreased blood pressure, which may well contribute to this protection [123].

Polymorphonuclear leukocytes play an important role in the development of ischemic damage by reducing microvascular blood flow, initiating thrombosis, and releasing free-oxygen radicals [122]. But anti-leukocyte interventions cannot attenuate ischemic brain damage [122]. ETs enhance the expression of intracellular adhesion molecule-1 and IL-8 on brain capillary endothelial cells via ET_A [61] suggesting the possibility of polymorphonuclear leukocytes being implicated in ET-induced brain tissue damage.

Inactivation of vascular ET_B accelerates pathological vascular remodeling in which gene expression of ET-1 mRNA is comparably increased, whereas tissue basal NO level is significantly decreased [124]. Gene expression of ET-1 and endothelial NO synthase is reciprocally regulated by flow or shear stress in cultured endothelial cells [125]. Therefore, it may be suggested that decreased CBF initially induces an increased ET-1 expression and a decreased NO production, resulting in an imbalance between ET-1 and NO levels in ischemic lesions [124]. Furthermore, failure of ET_B-mediated NO release cause a significant decrease in tissue NO_x level and consequently leads to pathological aggravation of ischemia [125]. In addition, ET_B blockade following ischemia modulates glial scar formation and may provide a more permissive substrate for neuronal survival and regeneration [97]. One important question remains whether ET receptors also directly or indirectly drive astrocyte changes following neuronal damage. In addition, ET acting at astrocyte ET_B can have a significant worsening impact on the glial environment after brain damage favoring the use of a mixed ET_A- and ET_B-antagonist. Furthermore, ET_B expressed by astrocytes is accessible to pharmacological manipulation, as either stimulation or antagonism of this receptor subtype significantly alters the damage-induced hypertrophy and number of astrocytes expressing this receptor.

Endothelin and their role in cerebral vasospasm

Synthesis of ET-1 is stimulated by different vasoactive agents, including oxyhemoglobin and thrombin, which can be liberated during posthemorrhagic clot lysis [126]. There is considerable evidence supporting the concept of a causative role of up-regulation of ET_B in association with enhanced functional response to ET-1 in the development of vasospasm after SAH: early plasma ET correlate with large artery vasospasm, symptomatic ischemia, and ischemic lesions as demonstrated with state-of-the-art imaging methods in the later course of the disease [2, 127]. ET-1 plasma level may represent therefore a biomarker of temporal course and severity of later ischemia, and reflect concentrations that may be much higher in local circulation [2]. In addition, serum ECI-1 activity is later increased demonstrating possibly endothelial damage [127].

A randomized placebo-controlled study on efficacy of $ET_{A/B}$ antagonist in treating SAH demonstrated an almost significant effect on the outcome in patients who had already received other modalities of treatment to prevent delayed ischemia [127]. It is conceivable, however, that low concentrations of ET-1 could augment effects of other agents liberated from the blood clot and, thus, substantially contribute to pathological contraction and cerebral ischemia observed in clinical vasospasm.

The future

Various effects of ET in vascular and non-vascular brain tissue have been discussed in the context of ischemic damage [125]. However, the mode of action of ET in the CNS is a continuum from physiologically relevant neuromodulatory effects at low concentrations [128] through reversible hypermetabolism at higher doses [125], to permanent neurotoxic damage at very high concentrations [129]. The underlying neurochemical mechanisms do not have necessarily to be different. For example, while it is clear that physiological activation of ET receptors induces a rise in $[Ca^{2+}]_i$ [130], supraphysiological levels of Ca^{2+}-influx have been associated with neurotoxic events [84]. Alternatively, it is possible that a low-dose, physiological action of ET is mediated by a direct effect on dopaminergic terminals [131], whereas high-dose effects also involve other mechanisms, such as glutamate and NO release [125].

In the near future, there will be a requirement for intensive experimental investigation into the ability of pharmacological tools to protect the development of ischemic stroke [132]. By this effort, the

importance of the role of ET in the physiological function of the cerebravascular system and its impact on a variety of neurological diseases may be better clarified. Therefore, reducing synthesis and release of ET-1 from endothelial and parenchymal (i.e. glial and neuronal) cells may prove worthwhile alternative or adjunctive therapeutic options in human stroke, as strongly suggested by animal experiments. With recent development of ECE inhibitors or new and more selective ET receptors antagonists, research tools to address this question may better have become available.

Conclusions

Discovery and development of specific endothelin-receptor antagonists have rapidly advanced our understanding of the biology of this family of vasoactive peptides. Use of these antagonists has allowed investigators to begin to discern important roles for ET in physiology and pathology of the vasculature. Future work should provide a clearer understanding of the complex nature and role of ET-1 in ischemia. The finding of a correlation of ET-1 plasma levels with lesion size suggests that ET-1 levels may be an indicator for the amount of damaged brain.

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