Mechanism of the delay phenomenon: tissue protection is mediated by heme oxygenase-1

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Mechanism of the delay phenomenon: tissue protection is mediated by heme oxygenase-1. Am J Physiol Heart Circ Physiol 287: H2332–H2340, 2004. First published June 24, 2004; doi:10.1152/ajpheart.01109.2003.—Induction of the “delay phenomenon” by chronic ischemia is an established clinical procedure, but the mechanisms conferring tissue protection are still incompletely understood. To elucidate the role of heme oxygenase-1 [HO-1 or heat shock protein-32 (HSP-32)] in delay, we examined in the skin-flap model of the ear of the hairless mouse, 1) whether chronic ischemia (delay) is capable to induce expression of HO-1, and 2) whether delay-induced HO-1 affects skin-flap microcirculation and survival by either its carbon monoxide-associated vasodilatory action or its biliverdin-associated anti-oxidative mechanism. Chronic ischemia was induced by transection of the central feeding vessel of the ear 7 days before flap creation. The flap was finally raised by an incision through four-fifths of the base of the ear. Microcirculatory dysfunction and tissue necrosis were studied with the use of laser Doppler fluxmetry and intravital fluorescence microscopy. HO-1 protein expression was determined with Western blot analysis. Seven days of chronic ischemia (delay) induced a marked expression of HO-1. This was paralleled by a significant improvement (P < 0.05) of microvascular perfusion and a reduction (P < 0.05) of flap necrosis when compared with nondelayed controls. Importantly, blockade of HO-1 activity by the vitamin E analog trolox after blockade of HO-1 to mimic exclusively the anti-oxidative action of the heat shock protein did not restore the HO-1-associated microcirculatory improvement and only transiently attenuated the manifestation of flap necrosis. Thus our data indicate that the delay-induced protection from tissue necrosis is mediated by HO-1, predominantly through its carbon monoxide-associated action of adequately maintaining nutritive capillary perfusion.

Tissue oxygen supply. These include also the induction of the “delay phenomenon,” i.e., conditioning of the tissue by moderate chronic ischemia (CI) to increase tolerance against a subsequent, potentially lethal ischemic injury (12).

Although these studies have shown improvement of flap survival independent of the nature of the conditioning procedure, pharmacological interventions may vary in effectiveness to enhance flap survival and may in general not achieve the degree of tissue protection, which is observed after surgical conditioning procedures (22, 31). Thus surgical ischemic conditioning has been suggested the predominant experimental and clinical method to improve flap survival (6, 32).

Surgical ischemic conditioning of the tissue may be done by the classic ischemic preconditioning or by inducing the delay phenomenon. Ischemic preconditioning consists of a short but complete ischemia, which is performed intraoperatively for a few minutes by clamping the main feeding vessels just before final flap creation (1). In contrast, the delay phenomenon is induced by moderate CI through ligation of individual feeding vessels several days before flap creation (39). Despite the fact that the latter procedure has been used for many years in clinical practice, the mechanisms of the delay phenomenon in protecting tissue against necrosis are still poorly understood. Previous studies (3, 12, 46) have indicated that the delay acts via maintenance of an adequate microcirculation and adaptive metabolic changes at a cellular level, resulting in improved tolerance against a subsequent ischemic insult. However, the molecular mechanisms of protection of tissue by the delay phenomenon have not been elucidated yet.

Heme oxygenase (HO)-1 may play an important role in the delay-mediated protection of tissue. This microsomal enzyme catalyzes the initial and rate limiting reaction in heme catabolism, i.e., the oxidative cleavage of the α-meso-carbon bridge of β-type heme molecules to yield equimolar quantities of biliverdin-IXα, carbon monoxide (CO), and iron (47). Biliverdin-IXα reduced to bilirubin acts as a potent antioxidant, which protects cells against oxidative stress (26, 42). CO, a further product of the pathway, acts like nitric oxide, on soluble guanylyl cyclase, increasing cGMP, a second messenger involved in the regulation of multiple cell functions, and thereby contributing to the regulation of vascular tone (16, 29). Heme oxygenase exists in three isoforms, i.e., HO-1, HO-2, and HO-3 (8). Among the three isoforms, HO-1 can be induced substantially and has been identified as the major 32-kDa heat shock (stress) protein HSP-32 (25). Like other stress proteins, HO-1 can be induced by a variety of apparently disparate...
stimuli, most of which are linked by their ability to provoke oxidative stress (8).

As the result of the above-mentioned properties of the enzyme system, it is conceivable to hypothesize that the protective effect of the delay phenomenon is mediated by induction of HO-1. Therefore, we investigated whether 1) conditioning of a skin flap by CI induces HO-1 expression, 2) the activity of this enzyme improves flap survival, and 3) vasoactive or anti-oxidative actions of the enzyme system mediate delay-associated tissue protection.

MATERIALS AND METHODS

Animals. The ear of the homozygous hairless mouse (skh-1) of both gender (8–10 wk old, 22–26 g body wt) was used as the skin flap model in this study (4). The studies were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1985), and the study protocol was approved by the Regional Review Board for the Care of Animal Subjects.

The mouse ear consists of a single layer of cartilage sandwiched between two full dermal layers of skin (overall thickness 300 μm) and is completely hairless, which makes it ideal for intravital microscopic studies. Blood supply is provided by three vascular bundles, entering the ear at its base and forming an interconnecting capillary network at the periphery (5).

The animals were housed in individual cages and had access to standard laboratory chow (Altromin) and water ad libitum. For induction of the delay phenomenon and creation of the skin flaps, the mice were anesthetized with an intraperitoneally administered mixture of ketamine and xylazine (90 and 25 mg/kg body wt).

Induction of the delay phenomenon and creation of the skin flaps. After anesthesia, the ear to be investigated was gently extended over a microscopic slide embedded into a Plexiglas pad. The delay phenomenon was provoked by local CI induced by transection of the central feeding vessel of the ear 7 days before flap creation (3).

To create an arterial pattern skin flap, the ear was incised through four-fifths of its base, rendering it dependent solely on the anterior vessel for its nourishment. The creation of the skin flap is associated with nutritive perfusion failure, and, within a 5-day observation period, development of tissue necrosis encompassing ~40% of the overall area of the ear (Fig. 1) (4).

Laser Doppler fluxmetry. Microvascular perfusion in the proximal, central and distal part of the flap was assessed by laser Doppler fluxmetry and microscopic analysis (model PF3, Perimed). The setup consists of a laser diode (3 mW, 810-nm emission monochromatic wave length) and a fiber optic sensor, which could be positioned manually over the tissue. The results were obtained by averaging the measurements of five different areas within each part of the ear, thus minimizing the spatial heterogeneity, well known even under conditions of normal tissue perfusion (28).

Intravital fluorescence microscopy. For the in vivo microscopic procedure, the ears were flattened on the Plexiglas pad. In the proximal, central, and distal part of the flap, microcirculatory parameters and tissue necrosis were analyzed by intravital fluorescence microscopy with the use of a Leitz Orthoplan microscope. Microscopy was performed at a constant room temperature of 23°C. Contrast enhancement for visualization of the microcirculation was achieved by injection of 0.1 ml 5% FITC-dextran 150,000 (Sigma Aldrich; Deisenhofen, Germany) into a tail vein. The dye binds neither to endothelial cells nor to individual blood cells and does not extravasate under physiological conditions. Because of its high molecular weight, it remains within the intravascular space for an ~4-h period and is then cleared from the circulation by the liver and the kidney. All measurements were done after the ears were embedded on a Plexiglas pad and fixed with an oxygen-impermeable foil.

The microscopic epo-illumination setup included a 100-W mercury lamp and a Ploemopak illuminator equipped with an I2 blue filter (450–490 nm excitation/515 nm emission wave lengths). The movement of the microscope platform in x/y-directions was achieved manually, which allowed measurements in five positions in each part of the ear, comparable to the laser Doppler fluxmetry analysis. Microscopic images were recorded by a charge-coupled device video camera (CF8/1 FMC, Kappa; Gleichen, Germany) and were stored on videotape for subsequent off-line evaluation.

Analysis of microcirculation and tissue necrosis. All microcirculatory parameters, i.e., capillary diameter, functional capillary density, red blood cell velocity (RBV), volumetric blood flow (VBF), and tissue necrosis were quantified off-line by analysis of the videotaped images using a computer-assisted image analysis system (CapImage; Zeintl Software; Heidelberg, Germany). RBV was assessed by the line shift method (2). Necrosis was quantified by determining the ratio of tissue area with nonperfused capillaries divided by the total tissue area of the ear (4). Capillary diameters were measured perpendicularly to the vessel path (in μm) only in red blood cell-perfused capillaries. Functional capillary density was defined as the length of all red blood cell-perfused capillaries per area of skin surface (cm/cm²) (28). Individual VBF was calculated in capillaries from RBV and vessel cross-sectional area (πr²) according to the equation VBF = RBV × πr², assuming cylindrical vessel geometry. Both RBV and VBF are presented as a percentage of the baseline to demonstrate the changes during the observation time.

Western blot analysis of HO-1. Pooled protein (100 μg/lane) from ear tissue homogenates (4 ears/group) were subjected to SDS-PAGE under denaturing conditions and transferred to polyvinylidene difluoride membranes. The blot was probed with polyclonal rabbit anti-rat HO-1 antibody (StressGen Biotechnologies; Sidney, BC, Canada) after blocking of unspecific binding sites with a mixture of 5% nonfat dry milk in 20 mM Tris(hydroxymethyl)aminomethane, pH 7.5, 0.5 M NaCl, and 0.1% Tween 20. A horseradish peroxidase-linked donkey anti-rabbit secondary antibody (dilution: 1:10,000) was used to detect protein-antibody complexes with a chemiluminescent reaction-enhanced chemiluminescence Western blot analysis system; Amersham Buchler; Braunschweig, Germany) according to the manufacturer’s instructions.
Immunohistochemistry of HO-1. Cell-type-specific expression and spatial pattern of HO-1 induction was studied in paraffin-embedded dewaxed sections essentially as described previously (7). Briefly, after incubation of sections in 3% H2O2-methanol to block endogenous peroxidase activity, slides were incubated with normal goat serum, followed by the primary HO-1 antibody used for Western blot analysis (dilution 1:200) at 37°C for 1 h. A biotinylated goat anti-rabbit antibody was used as secondary antibody for streptavidin-biotin complex peroxidase staining; 3,3′-Diaminobenzidine and 3% NiCl2 were used as chromogens, and slides were counterstained with hematoxylin.

Chemicals. Tin-protoporphyrin-IX (SnPP-IX, Porphyrin Products; Logan, UT) was dissolved by mixing SnPP-IX with sodium bicarbonate 8.4% and phosphate-buffered saline to achieve a final concentration of 5 μmol/ml. The solution was stored at a maximum temperature of 8°C in a light-protected tube until usage for not >1 h. The water-soluble vitamin E analog Trolox (Sigma Aldrich; Steinheim, Germany) was dissolved by mixing 97% 6-hydroxy-2,5,7,8-tetramethylchroman-2-carbonsacid and 0.9% saline. All solutions were freshly prepared at the day of the experiment according to the directions for use given by the manufacturer.

Experimental protocol. Tissue necrosis and microvascular perfusion in the proximal, central, and distal part of the flaps were determined before conditioning, immediately after skin flap creation, and, thereafter, daily over a 5-day period. After baseline measurements, the induction of CI or flap creation was performed as described before. After the induction of CI, animals of the first group were not further pretreated (CI, n = 8). To study the role of HO-1 in this model, animals of the second group received the false substrate SnPP-IX (50 μmol/kg body wt ip; CI+SnPP-IX; n = 8) to block HO-1 activity before the induction of CI and directly before flap creation. To test whether the antioxidative action of HO-1 is responsible for tissue protection, animals of the third group received beside SnPP-IX the vitamin E analog trolox (6 mg/kg body wt iv, every 12 h; CI+SnPP-IX+Trolox, n = 8) at the time of flap creation. Animals with flap creation but without any treatment served as controls (Control, n = 8). The substance-specific effects of SnPP-IX and trolox on microcirculation and flap necrosis were additionally assessed in animals without chronic ischemic conditioning (Control+SnPP-IX and Control+Trolox, both n = 8).

Statistical analysis. Data are given as means ± SE. After the assumption of normality was proven, comparison between the experimental groups was performed by one-way ANOVA, followed by the appropriate post hoc comparison test. P < 0.05 was considered to indicate significant differences.

RESULTS

Immunohistochemistry. Immunohistochemical analysis of expression pattern of HO-1 at the time of flap creation showed a distinct HO-1 protein expression in those animals, which were conditioned by CI (CI+SnPP-IX, CI+SnPP-IX+Trolox), whereas HO-1 expression was lacking in nonconditioned controls (Fig. 2).

Time course of HO-1 protein expression. Immunoreactive protein expression was additionally analyzed by Western blot analysis, which confirmed the results made by immunohistochemistry. At the day of flap creation HO-1 protein expression was detected only in conditioned animals (CI, CI+SnPP-IX, and CI+SnPP-IX+Trolox), but not in nonconditioned controls. During the time course after flap creation, HO-1 protein expression increased in all groups and reached the highest values at the end of the 5-day observation period (Fig. 3). The treatment of CI-conditioned animals with SnPP-IX did not affect the expression of HO-1. The addition of Trolox at the time of flap creation in these animals (CI ± SnPP-IX ± Trolox) only slightly reduced overall HO-1 expression at day 5, whereas Trolox application in nonconditioned controls markedly depressed the flap creation-associated HO-1 expression (Fig. 3).

Tissue necrosis. During the entire observation period, induction of delay, i.e., conditioning by CI, resulted in a significant reduction of tissue necrosis after flap creation when compared with that of nonconditioned controls (day 5: CI: 16 ± 3%; Control: 41 ± 3%, P < 0.05). Blockade of the HO-CO pathway by SnPP-IX in the CI-conditioned animals almost completely blunted the delay-associated protection, resulting in an amount of tissue necrosis similar to that of nonconditioned controls (day 5: 36 ± 5%, P < 0.05 vs. CI). Additional administration of Trolox after HO-1 blockade led to a significant reduction of flap necrosis only until day 3 after flap creation when compared with that of nondelayed controls (day 3: CI+SnPP-IX+Trolox: 19 ± 3%; Control: 33 ± 4%, P < 0.05), whereas at day 5, the amount of nonperfused tissue was also significantly higher than that in nontreated, solely CI-conditioned animals (day 5: 32 ± 4%, P < 0.05 vs. CI) (Fig. 4).
Microcirculation. Pretreatment with CI significantly reduced microvascular perfusion failure after flap creation, particularly in the distal, most vulnerable part of the flap. Blockade of the HO-CO pathway by SnPP-IX blunted CI-associated amelioration of perfusion, resulting in perfusion conditions comparable to controls. Additional treatment with Trolox did not affect the microcirculation compared with mice receiving SnPP-IX only.

In the proximal part of the ear, flap creation resulted in a minor capillary dilation (Fig. 5A), which was associated with a slight alteration of microvascular perfusion, including red blood cell flux (Fig. 6A), as determined by laser Doppler flowmetry, and functional capillary density (Fig. 7A), capillary RBV (Fig. 8A), and capillary VBF (Fig. 9A), as analyzed by intravitral microscopy. CI-conditioned animals showed also minor capillary dilation (Fig. 5A) and a decrease of functional capillary density (Fig. 7A); however, these animals maintained overall red blood cell flux (Fig. 6A) and capillary RBV (Fig. 8A), resulting in a higher individual capillary VBF when compared with that of nonconditioned controls (Fig. 9A).

Blockade of HO-1 by SnPP-IX completely abrogated these beneficial effects of delay, and additional administration of Trolox did not affect the SnPP-IX-induced abrogation of microcirculatory protection (Figs. 6A, 8A, and 9A).

In the central part of the ear, flap creation further deteriorated the ears’ microcirculation, whereas CI-induced delay tended toward protective maintenance of an adequate microvascular perfusion (data not shown).

In the distal part of the ear, flap creation induced marked capillary dilation (Figs. 5B and 10), which was associated with a massive alteration of microvascular perfusion to <10% of baseline, including red blood cell flux (Fig. 6B), functional capillary density (Fig. 7B), capillary RBV (Fig. 8B), and capillary VBF (Fig. 9B). Most strikingly, CI (delay) further pronounced capillary dilation (Figs. 5B and 10D) and was effective to significantly attenuate the decrease of red blood cell flux (Fig. 6B), functional capillary density (Fig. 7B), and capillary RBV (Fig. 8B). This resulted in complete maintenance of capillary VBF (Fig. 9B). Blockade of HO-1 by SnPP-IX abrogated capillary dilation (Figs. 5B and 10F) and completely blunted the CI-associated attenuation of microvas-
cular perfusion failure, including red blood cell flux (Fig. 6B), functional capillary density (Fig. 7B), capillary RBV (Fig. 8B), and thus capillary VBF (Fig. 9B). Trolox did not restore the SnPP-IX-mediated loss of protection from microcirculatory dysfunction (Figs. 5B, 6B, 7B, 8B, 9B, and 10H).

In nondelayed controls, additional SnPP-IX (Control+SnPP-IX) or Trolox (Control+Trolox) treatment did not reveal any modulation of microcirculatory disorders induced by the flap creation procedure, except of abrogation of capillary dilation by SnPP-IX (data not shown).

**DISCUSSION**

The major finding of the present study is that CI (delay)-induced protection against skin flap microcirculatory dysfunction and necrosis is mediated by the heat shock protein HO-1, predominately through its CO-associated vasodilatory action.

**CI strongly induces gene expression of HO-1.** Since 1968, when the first heme oxygenase (HO) system was detected (47), strong efforts have been made to characterize the function of this enzyme. HO represents the most efficient system to catalyze the oxidative cleavage of heme to equimolar amounts of biliverdin, CO, and iron (8, 47). Meanwhile, three isoforms of HO have been identified. The noninducible HO-2, constitutively expressed in most tissues, including the brain, testis, and liver (7, 26), seems to be of particular importance for cell integrity and cell function. The recently discovered, barely known isoform HO-3, which is expressed at a very low level, seems to be responsible for intracellular binding and transport of iron (15, 27). The isoform HO-1 is an enzyme of particular interest because of its identification as HSP-32 (25, 38). Like all other stress proteins, HO-1 is inducible by a variety of stress factors, e.g., heme (39), heat (38), hyperoxia but also hypoxia.
HO-1 is expressed in all tissues analyzed (26). The present study demonstrates the induction of HO-1 in skin tissue by chronic hypoxia, probably triggered by the hypoxia promoter (13) on the 5′ region of the HO-1 gene. Nonetheless, the generation of oxidative stress rather than the availability of distinct promotors seems to be the common trigger for HO-1 gene expression after exposure to potential inducers (8, 36). Thus production of oxygen radicals leads to an activation of oxygen-dependent transcription factors, such as activator protein-1 and NF-κB. Both are predominantly involved in the regulation of HO-1 gene transcription (8), supporting the widespread inducibility of this enzyme. This pathway may also be responsible for the induction of HO-1 expression by CI, which is thought to be the inducer of the delay phenomenon. Chronic hypoxia generates oxidative stress (14), which mediates HO-1 gene expression by oxygen-dependent transcription factors (8, 36). This view is supported by the present observation that flap creation without chronic ischemic conditioning also induced HO-1 expression over time, indicating that this procedure also resulted in tissue hypoxia, which activated HO-1 de novo expression.

**HO-1 improves skin flap viability.** Flap transfer is a widely used technique in plastic and reconstructive surgery. Although strong efforts have been made to understand flap pathophysiology (23) to develop novel strategies for improvement of flap viability, there is no distinct approach available to prevent flap (36), and has been shown to be expressed in all tissues analyzed (26).

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...in vivo and indicate that the protective effect of the delay phenomenon is mediated by the action of HO-1, which catalyzes the cleavage of heme to CO, biliverdin, and free iron. CO and its action on capillary pericytes may play a major role in this delay-associated protection. It is well known that capillaries are associated abuminally with pericytes (21), which pro-trude primary cytoplasmic processes running along the capillary axis. From these primary processes, lateral processes arise, which completely encircle the capillaries and form tight connections to the endothelial cells through multiple contact points (10, 17, 21, 37, 40). These pericytes contain muscle cytoskeletal proteins, in particular, α-smooth muscle actin (41), which are thought to regulate microvascular blood flow (21). In liver tissue, in vivo studies have shown that Ito cells, which are the sinusoid (liver capillary)-associated pericytes (44), are responsible for CO-mediated blood flow regulation in sinusoids, because blockade of CO resulted in sinusoidal constriction at the site of the Ito cells (43). This view was additionally confirmed in that the addition of CO, which is known to increase intracellular cGMP levels (29, 33), or the direct application of a cGMP analog resulted in attenuation of sinusoidal constriction (43). On the basis of this knowledge, increased CO levels, generated by elevated HO-1 activity, may explain the increase in capillary diameter, which ameliorates the blood supply of stress-exposed tissue, as indicated by increased red blood cell flux and VBF. In addition, there is evidence that HO-1 positively influences blood rheology (11), which could result in higher RBV because of unhindered flow. By this, tissue viability increases because of improved nutritive supply and better elimination of toxic residues of oxidative stress.

Biliverdin is subject to further degradation to bilirubin by the cytosolic enzyme biliverdin reductase (8, 26). It acts as an antioxidant and is capable to scavenge free oxygen radicals that are thought to be primarily responsible for the tissue injury (42). The additional application of Trolox, which mimics the antioxidative action of bilirubin, transiently delayed the development of necrosis, however, without improving the flap-associated microcirculatory disorders. This may be because bilirubin, produced by HO-1, inhibits cell damage by scavenging oxygen radicals subsequently after the stress event, thus improving tissue viability. Iron, the last product of heme cleavage, acts as a pro-oxidant like other transition metals and catalyzes the formation of reactive -OH by the Fenton or Haber-Weiss reaction. These radicals are responsible for activation of cell death programs (8), which aggravate stress-induced cell damage. It seems to be of particular importance to eliminate free iron from the stressed tissue to maintain cellular integrity after the stress event. To enable this process, an additional expression of ferritin, the iron-binding protein, is induced simultaneously by HO-1 (8, 18).

The results of the present study now demonstrate delay-induced maintenance of adequate nutritive capillary perfusion...
protection under critical ischemic conditions (20). The present study, however, now indicates a major role of HO-1 for delay-induced improvement of flap survival, probably by reducing microcirculatory failure within the critically endangered tissue portions. This conclusion is based on the fact that inhibition of HO-1 by SnPP-IX almost completely blunted both delay-induced preservation of microcirculation and improvement of flap survival.

Delay-associated tissue protection is obtained by HO-1-mediated attenuation of microcirculatory dysfunction. The enhanced HO-1 activity may provide tissue protection by CO-mediated vasodilation or biliverdin-associated anti-oxidative actions (35). To elucidate the role of a solely anti-oxidative treatment, the vitamin E-analog Trolox was given after excluding the action of both CO and biliverdin by blockade of HO-1 with SnPP-IX. Although Trolox may not exactly mimic the action of biliverdin, its effects allow us to evaluate the role of reactive oxygen species in mediating flap microcirculatory dysfunction. As demonstrated in the present study, anti-oxidative Trolox treatment after selective blockade of HO-1 reduced necrosis only until day 3 after flap creation and did not affect the manifestation of microcirculatory disorders. This result indicates that oxidative stress may play some role in the development of initial flap necrosis, and that anti-oxidative therapy is capable to counteract this early oxidative stress-induced cell damage. However, it also demonstrates that the initial oxidative stress does not determine the final amount of flap necrosis, and that the overall protection from flap necrosis by chronic ischemic conditioning (delay) is mediated by HO-1 not through its anti-oxidative but rather through its vasodilatory action, preventing microvascular perfusion failure.

In summary, we conclude that the induction of HO-1 is an important mechanism by which the delay phenomenon provides protection to critically endangered tissue, and that the improvement of flap survival is mainly due to the efficacy of HO-1 to adequately maintain nutritive capillary perfusion.

GRANTS

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