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Refractoriness to the Effect of Endothelin-1 in Porcine Ciliary Arteries

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Abstract

Purpose: Endothelin-1 (ET) is an important molecule in vascular physiology. After an acute stimulation with ET, vessels are to some extent temporarily refractory to further stimulation. However, few details are known about this phenomenon. The aim of our study was to verify the existence of refractoriness in ophthalmic ciliary arteries and, if present, to analyze its time course.

Methods: Twenty freshly isolated porcine ciliary arteries were placed in a myograph system to measure isometric forces. Each vessel was stimulated with 10^-7 M ET twice. The experiment was performed in 5 groups of vessels, which differed in the time interval between the initial and the second stimulation with ET. The intervals were 15 min, 30 min, 1 h, 2 h, and 4 h, respectively.

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Conclusions: Our experiment with isolated porcine ophthalmic ciliary arteries revealed a refractoriness phase to ET after an acute stimulation with ET. This refractoriness was transient and disappeared after 4 h. The lowest response was observed in the group of vessels re-exposed 15 min after the first stimulation.

Introduction

Endothelin, identified in 1988 by Yanagisawa et al.,1 is one of the strongest known endogenous vasoconstrictors. The predominant isoform in the endothelin family is endothelin-1 (ET). Under physiological conditions, ET is mainly synthesized by vascular endothelial cells. However, in hypoxia or other pathological conditions, ET can also be produced by other cells.2-4 In humans, the effects of ET are mediated by 2 types of ET receptors: the type-A receptor (ETA) and the type-B receptor (ETB). ETA receptors are found on vascular smooth muscle cells, and their stimulation leads to marked and sustained vasoconstriction by increasing cytoplasmatic calcium. ETB receptors can be found on both endothelial cells and smooth muscle cells. ETB receptors on endothelial cells mediate the release of vasodilatory nitric oxide and/or prostacyclin; however, stimulation of ETB receptors on smooth muscle cells provokes vasoconstriction.5

Together with other vasoactive molecules and the autonomic nervous system, ET regulates vascular tone. Several factors modulate the production of ET, including shear stress,6 hypoxia-inducible factor-1-alpha,7 angiotensin II,8,9 adrenaline,10 inflammatory cytokines11,12 and others.

It is obvious that any malfunction in this system locally or systemically can have pathophysiologic implications, and a relative increase in ET can lead to vasoconstriction or even vasospasm. Indeed, ET raises blood pressure13 and induces vascular and myocardial hypertrophy,14,15 both contributors to increased cardiovascular morbidity and mortality.16 ET has been shown to be involved in the pathogenesis of many diseases.5

In the eye, the regulation of blood flow is different in distinct vascular beds. Retinal circulation is mainly regulated by endothelium-derived vasoactive factors, while choroid circulation is predominantly regulated by the autonomic nerve system.15 Optic nerve head circulation is, like that of the retina, regulated by endothelial cells, but it is also under the direct influence of circulating hormones (including ET). These circulating hormones diffuse from fenestrated capillaries of the choroid into the optic nerve head and reach the vascular smooth muscle cells directly, by-passing the blood-retinal barrier.17,18 This is the reason why an increased level

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of ET in the circulating blood (as can be observed in many different autoimmune diseases such as multiple sclerosis) has a major effect on optic nerve head circulation but no or only a minimal effect on retinal or brain circulation. An increased level of ET seems to be implicated in the pathogenesis of many ocular diseases, particularly retinal vein occlusion,20,21 glaucoma,22 diabetic retinopathy,22 and giant cell arteritis.23

Although ET obviously plays an important role, many basic aspects of its function are still poorly understood, including the question of the dose-response relationship. An acute rise in ET concentration may have a completely different effect from a chronic increase.

The mode of action of ET is different from other vasoconstrictors like adrenaline or angiotensin II. Once ET binds to its receptor, it will barely dissociate again.24 After binding, ET is rapidly internalized together with its receptor.25 Such an internalization of the ET-ET receptor complex influences local ET concentration to some extent and has a major impact on receptor density and thus ET sensitivity.

Although tachyphylaxis with ET was already observed in rat aortic rings in 1993,26 this phenomenon was not taken into account in later in vitro and ex vivo study designs. On the contrary, in many studies investigating the effect of ET and its blockers on vascular function, vessels were exposed to stepwise incremental concentrations of ET. If the vasculature is refractory to ET, the results of such studies should be interpreted with caution, especially when high concentrations of ET are studied. Information about refractoriness is of relevance for physiology, pathophysiology, and pharmacology, especially in light of the fact that ET and its receptors are promising targets for future drugs.

The purpose of the present study was a) to test whether refractoriness indeed exists in ocular vessels and b) to estimate its time course. By working on isolated vessels, the effect of ET is separated from the influence of other regulatory systems, like the autonomic nerve system or neurovascular coupling.

Methods

Preparation of vessels

In adherence with the Association for Research in Vision and Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research, porcine eyes with surrounding tissue were obtained from a local slaughterhouse immediately after death and transported in cold modified Krebs-Ringer solution (NaCl 118 mMol, KCl 4.7 mMol, KH2PO4 1.2 mMol, MgSO4 1.2 mMol, NaHCO3 25 mMol, EDTA 0.026 mMol, CaCl2 2.5 mMol, Glucose 11.1 mMol).

Ciliary arteries were dissected under a microscope (Wild M3C; Wild Heerbrugg AG) and cut into 2-mm segments. Arterial rings were mounted in specially designed organ chambers for small vessels (myograph system27). In an organ chamber, two 40-μm tungsten wires were passed through the vessel's lumen and attached to a force transducer for isometric force measurements (Multi Wire Myograph System 610M; Danish Myo Technology). Mounted vessels were immersed in the myographs' organ chambers filled with Krebs-Ringer bicarbonate solution (37°C; 95% O2, 5% CO2) and stretched in a stepwise manner until the optimum passive tension was reached. The optimum passive tension was defined as the level of vascular wall tension at which contractions to 100 mM potassium chloride (KCl) became maximal. The vessels were then washed out with modified Krebs-Ringer solution before the following experimental protocols were conducted.

Experimental protocols

Each of 20 ciliary arteries was stimulated with ET twice. Quiescent vessels were first exposed to 10−4 M ET, then washed out with modified Krebs-Ringer solution, and then exposed to 10−4 M ET again. Five series of experiments were performed, which differed in the time interval between the initial and the second stimulation with ET: 15 min, 30 min, 1 h, 2 h, and 4 h, respectively. At the end of each experiment, vessels were exposed to 100 mM KCl to establish that they were still responsive to vasoconstrictor agents. In each series of experiments, 4 porcine ciliary arteries of 4 animals were used (one vessel per experiment).

Drugs and statistical analyses

ET was purchased from Sigma-Aldrich Chemie GmbH. It was dissolved in distilled water. Concentrations are expressed as final molar concentrations in the organ chambers.

Results are given as ratio ETsec/ETini, where ETsec represents the response to the second stimulation with ET and ETini represents the response to the initial stimulation with ET. To explore the ratio ETsec/ETini descriptive statistics including mean and standard deviation were calculated. A graphical description using boxplots was assessed. To compare ETsec/ETini across all time points, a linear regression model was performed. All ratios were compared to 1. Results are also expressed as differences of means to 1 for each time point. Standard errors (SEs) and corresponding P values are also reported. P values and SEs were adjusted for multiple comparisons using the method of Dunnett.28 A P value < 0.05 was considered to be statistically significant. All analyses were done using the statistical package R version 12.2.29

Results

The vasoconstrictive response to re-exposure to ET was time-dependently reduced in comparison to the initial ET stimulation (Fig. 1, Table 1). The vasoconstrictive response to the first exposure to ET was arbitrarily defined as 1 U. 15 min, 30 min, 1 h, and 2 h after initial stimulation, the vasoconstrictive response to the second stimulation with ET was significantly lower than 1. The response was lowest at the 15 min time point with a mean response of 0.22 (ie, 22% of baseline response) and then slowly recovered to 0.29, 0.63, and 0.80 for the 30-min, 1-h, and 2-h time points, respectively. At the 4-h time point, the response reached an average of 0.9, a value that was no longer significantly different from the baseline value of 1.

At the end of the experiments, all vessels were still completely responsive to 100 mM KCl.

Discussion

The present study demonstrated that (1) after an acute stimulation with ET, vascular smooth muscle cells in the porcine ciliary arteries were temporarily partly refractory to further stimulation and (2) this refractory effect was...
observed in rat mesenteric arterial smooth muscle cells, in isolated guinea-pig ileum, and in human small bronchi. Concerning in vivo studies, however, the literature on the refractory phenomenon of ET is ambiguous. In anesthetized rats, the rapid development of desensitization to the hypotensive effects of ET was observed when repeated intravenous bolus injections of this peptide were given. However, in the same study, the vasoconstrictive effects of ET did not undergo a fading phenomenon upon successive administrations of ET in pithed rats. This discrepancy between ex vivo and in vivo studies may be due to the different doses and therefore concentrations applied. But it is also possible that vessels denervated and isolated from their surroundings behave differently.

The observation that repeated stimulation of receptors by their agonists can lead to transient refractoriness is not specific for ET. Such phenomena are particularly observed when receptors are stimulated with high doses and may protect cells from overstimulation and potential damage. The exact mechanism of refractoriness to ET is not known. One important component might be the internalization of the ET-ET-receptor complex. These complexes are rapidly internalized by receptor-mediated endocytosis. After internalization, ETA and ETB receptors are targeted to different intracellular fates. Whereas the ETA receptor follows the recycling pathway through the pericentriolar recycling compartment and then reappears at the plasma membrane, the ETB receptor is directed to lysosomes for degradation. Therefore, a longer lasting signaling response can be achieved upon ETA receptor stimulation. The cytoplasmic carboxyl-terminal tail of the ETA receptor is responsible for targeting this peptide to the recycling pathway. This motif is lacking in the carboxyl-terminal region of the ETB receptor, facilitating a divergent endocytic sorting of the ETA receptor and ETB receptor. The time of internalization until reappearance of the receptors may correspond to the refractoriness.

However, receptor recycling may not be the only regulatory mechanism accountable for the refractory phenomenon of ET. ET receptors belong to the G protein-coupled receptors (GPCRs) family. GPCR signaling receives negative feedback by G protein-coupled receptor kinases (GRK). ET signaling in arterial smooth muscle is tightly regulated by GRK2. The inhibition of GRK2 reduces the extent of ETA receptor desensitization, thus indicating that GRK2 may play an important role in the ET refractory phenomenon.

In addition, we cannot exclude that a certain component of the refractoriness is immanent to the experimental set-up. The vasoconstrictive response to the second ET stimulation might be reduced due to the fact that the resting tension at the time point of the second ET stimulation has not returned to the baseline levels yet. This, however, cannot explain the entire refractoriness observed in our study for the following reason: although the resting tension was still increased at the moment of the second ET stimulation, the total response (active response + resting tension) after the second ET stimulation was less than after the initial ET stimulation.

The observed refractoriness is most likely multifactorial. The purpose of the present study was to test the existence of refractoriness in an experimental set-up commonly used, particularly in drug studies. In such ex vivo experiments using a myograph system, vessels are often exposed to stepwise incremental concentrations of ET. Further studies are needed to explain the exact mechanism of this temporary

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**TABLE 1. Vasoconstrictive Response to the Second Stimulation with ET-1 in Relation to Baseline (ETini, Defined as 1.0) When Porcine Ciliary Arteries Were Re-exposed to ET-1**

<table>
<thead>
<tr>
<th>Time interval between baseline and re-exposure</th>
<th>Ratio ETsec/ETini</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min</td>
<td>0.22</td>
<td>0.11</td>
</tr>
<tr>
<td>30 min</td>
<td>0.29</td>
<td>0.09</td>
</tr>
<tr>
<td>1 h</td>
<td>0.63</td>
<td>0.17</td>
</tr>
<tr>
<td>2 h</td>
<td>0.80</td>
<td>0.08</td>
</tr>
<tr>
<td>4 h</td>
<td>0.90</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Results are presented as ratio ETsec/ETini (mean, SD, and P value) for each time point.

(ETsec=vasoconstrictive response to the initial stimulation with ET-1; ETini=vasoconstrictive response to the second stimulation with ET-1)

ET, endothelin-1; SD, standard deviation.

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**FIG. 1.** Twenty isolated ciliary arteries were stimulated with 10^-7 M endothelin-1 (ET) twice, on baseline condition and after a latency shown on the x-axis. The response to the initial ET stimulation (baseline) was arbitrarily defined as 1. The response to the second ET stimulation was expressed as the ratio ETsec/ETini and shown on the y-axis. (N=4 vessels for each time point). Results are presented as box plots. ETini=vasoconstrictive response to the initial stimulation with ET-1 (baseline value); ETsec=vasoconstrictive response to the second stimulation with ET-1 (re-exposure); Time=time interval between the initial and the second stimulation with ET-1.
desensitization of vascular smooth muscle cells to ET and to distinguish between the roles of the ET\textsubscript{A} and ET\textsubscript{B} receptors.

What about the clinical implication of these findings? The level of ET in the circulating blood is increased in many diseases, including ocular diseases such as retinal vein occlusion,\textsuperscript{19,20} diabetic retinopathy,\textsuperscript{22} giant cell arteritis,\textsuperscript{23} and retinitis pigmentosa.\textsuperscript{29} Less is known about the extravascular ET concentration. It is increased, for example, in the aqueous humor of glaucoma patients\textsuperscript{40} and is present in epiretinal membranes.\textsuperscript{31} Even less is known about the time course of ET concentrations in tissues such as retina. However, acute hypoxia—as it occurs in many clinical conditions—stimulates ET expression locally, and without refractoriness, it can lead to a vicious circle.

Furthermore, information about refractoriness to ET is of sure relevance for pharmacological vascular research. ET and its receptors are promising targets for future drugs.

What is the relationship between the concentration of ET used in our study and biological concentrations? The majority of ET produced in the endothelial cells is secreted abuminally, and only a small part is released into the lumen of the vessels. The resulting ET concentration in the circulating blood is in the range of pg/mL (1 pg/mL ET=0.4×10\textsuperscript{-12} M), whereas in the vascular wall, it is probably in the range of ng/mL (1 ng=0.4×10\textsuperscript{-9} M). However, little is known about the actual concentration in the extracellular space, between endothelial cells and smooth muscle cells. Even less is known about the corresponding concentration in the tissues under pathological conditions. Indeed, the concentration of ET used in our study may be higher than physiological concentration; however, it might be in the range reached under pathological conditions. We have chosen an ET concentration known to be within the range (upper half) of the concentration-response curve to ET in ex-vivo studies using a myograph system.\textsuperscript{43,44}

In conclusion, we definitively proved the existence of refractoriness to ET in ocular vessels, at least in ex-vivo experiments and, for the first time, established an approximate time course of this interesting phenomenon. While the biological relevance of this finding needs to be established, it should already be taken into account in studies involving repeated ET applications.

**Author Disclosure Statement**

The authors report no conflicts of interest. No competing financial interests exist.

**References**


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