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Release of the free nitric oxide radical (NO) and EDRF from endothelial cells

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Untersuchungen zur Freisetzung von Stickstoffmonoxid (NO) und EDRF aus Endothelzellen

Zusammenfassung: Die Freisetzung von Stickstoffmonoxid (NO) wurde aus kultivierten makrovaskulären Endothelzellen und isolierten Meerschweinherzen mit einem spezifischen spektrophotometrischen Assay gemessen. NO wurde unter basalen Bedingungen kontinuierlich sowohl aus kultivierten Zellen als auch aus dem isolierten Herzen in das koronarvenöse Effluat freigesetzt. Bradykinin (10^{-7} M) steigerte die NO-Freisetzungsrate in beiden experimentellen Modellen maximal zwei- bis dreifach. Der Beginn der NO-Freisetzung ging in allen Fällen dem Beginn der Vasodilatation voraus (< 15 s). Unsere Ergebnisse zeigen, daß 1. Endothelzellen NO direkt als freies Radikal freisetzen, 2. NO allein für die vasodilatatorischen Eigenschaften von EDRF verantwortlich ist, und 3. unter in vivo Bedingungen die endogene NO-Bildung quantitativ ausreichend ist, um an der Regulation des koronaren Gefäßwiderstandes teilnehmen zu können.

Schlüsselwörter: Stickstoffmonoxid (NO), Endothel, EDRF, Bradykinin, Koronarkreislauf

Summary: Release of nitric oxide (NO) from cultured macrovascular endothelial cells (EC) and from isolated perfused guinea pig hearts was measured with a specific spectrophotometric assay. Under basal conditions NO was continuously released from cultured cells and from isolated hearts into the coronary effluent perfusate. Bradykinin (10^{-7} M) increased rate of NO release maximally two- to threefold in both experimental models. Onset of NO release always preceded start of vasodilation (< 15 s). Our results provide evidence that under basal and bradykinin-stimulated conditions, 1) endothelial cells release nitric oxide as a free radical, 2) NO is solely responsible for the vasodilatory properties of EDRF and, 3) under in vivo conditions the endogenous formation of NO is quantitatively sufficient to influence the coronary vascular tone and thus, may play an important role in the regulation of coronary vascular resistance.

Key words: Nitric oxide; endothelial cell; EDRF; bradykinin; coronary circulation

Introduction

Within the last year evidence was provided by different laboratories that a parallel exists between the release of an endothelium-derived relaxing factor (EDRF) and endothelial formation of N-oxides (7, 12, 14). While these studies were carried out on cultured macrovascular endothelial cells (EC) recent reports demonstrated that one or more EDRF(s) may influence the tone of coronary resistance vessels (6, 16). None of these in vivo studies however have further characterized the chemical identity of the substances involved. In addition there are also reports describing different biological and chemical properties of EDRF and NO (11, 15).

Whether NO formation exclusively is responsible for the biological activities of EDRF requires an answer to the following questions: 1) Do endothelial cells directly release the free radical of NO or other precursors? 2) Does the onset of endothelial NO release precede the biological response of EDRF?

The aim of the present study was to directly compare the amount and time course of the release of the free nitric oxide radical and EDRF from cultured macrovascular cells and from the isolated perfused guinea pig heart.

Materials and Methods

Endothelial cells

Endothelial cells were isolated from porcine thoracic aorta and cultured in M199 medium plus 20 % fetal calf serum. Purity of culture was determined by immunological fluorescent staining with ac-LDL, phase-contrast, and scanning electron microscopy. Eight days after isolation cells were grown on microcarrier beads (Biosiolon, Nunc, $5\text{–}7 \times 10^6$ cells/g bead) (3), packed into a column and perfused at 2 ml/min with a HEPES-buffered Krebs-Ringer solution containing (mM): NaCl 140, KCl 4.0, $CaCl_2$ 1.8, $MgCl_2$ 1.03, NaH_2PO_2 0.42, glucose 5.0, pyruvate 2.0, HEPES 10.0 and indomethacine 0,01 (pH 7.4, 37°C).

Isolated guinea pig hearts

Guinea pigs, weighing 280–320 g, were stunned by a blow on the neck. Hearts were excised, perfused according to the Langendorff technique with the above mentioned medium (10 ml/min, equilibrated with 100% O_2) and electrically paced (285) bpm). Left ventricular systolic pressure (LVSP), dp/dt max and heart rate were documented on a Gould 2300 recorder. Left ventricular diastolic pressure was adjusted to 0 mm Hg. Hearts were accepted for further study only when 1) coronary perfusion pressure (CPP) was higher than 55 mmHg and 2) bolus injection of 50 µl adenosine (50 µM) decreased CPP by more than 20 mm Hg.

Measurement of nitric oxide

Endothelial NO release was continuously quantified in the effluent perfusate by a specific differencespectrophotometric assay (5, 8), which is based on the rapid oxidation of oxyhemoglobin (HbO_2) to methemoglobin (MetHb) by NO (4). Since HbO_2 traps the entire released NO in less than 100 ms (8) measurement of the extinction difference (λ_1 401 nm, λ_2 411 nm) in a flow-through cell with a double-beam double-wavelength spectrophotometer (Hitachi model 557, Perkin-Elmer) permitted the continuous assay of released NO. Concentrations of NO were calculated from the extinction coefficient determined under the conditions used in this study (38 mM^{-1} cm^{-1}).

Aqueous solutions of bovine hemoglobin were oxygenated and reduced by a molar excess of natriumdithionite and purified by gelfiltration (Sephadex G 25, Pharmacia) (5). Purity was controlled spectrophotometrically and by SDS - gelelectrophoresis.

Standards of aqueous NO solutions were prepared by saturation of deoxygenated water with purified nitric oxide gas, and further dilution. NO concentration of stock solutions was determined by means of HPLC (Merck-Hitachi, LiChrosorb-NH_2, Hibar) as nitrite ions.

Bioassay

An isolated perfused, endothelium-denuded, precontracted segment of rat aorta served as a stable bioassay for NO and EDRF. Aortas were perfused at constant flow with 2 ml/min and a pressure of 60 cm H_2O with the above mentioned HEPES-buffered Krebs-Ringer solution (10). Removal of endothelium by deoxycholic acid (0,75%, 15 sec) was verified by transmission and scanning electron microscopy and lack of responsiveness to acetylcholine (1 µM). Vessel diameter was measured by a pair of piezoelectrical crystals (4 MHz, detection limit of diameter changes: 2 µm).

Experimental protocols

Parallel measurement of EDRF and NO release in two columns, packed with EC covered microcarriers, was performed under identical experimental conditions with EC of the same cell batch. In the case of NO measurement perfusion medium was supplemented with 4 µM HbO_2 (Fig. 1).

In isolated guinea pig hearts dose-response curves for bradykinin (BK) were determined in the presence and in the absence of hemoglobin. The brady-

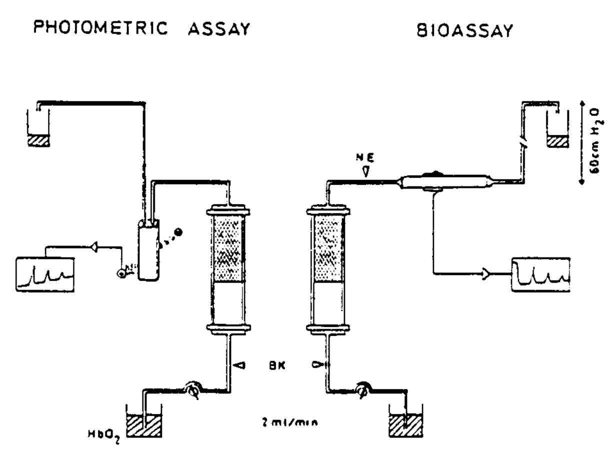

Fig. 1. Cultured EC grown on microcarriers beads were perfused in a column (2 ml/min) and stimulated with bradykinin (BK). Release of EDRF was continuously determined in the bioassay. Changes in NO release were determined with a double-wavelength-double-beam spectrophotometer. Measurement of both parameters was performed in parallel with two columns under identical experimental conditions. Only EC of the same cell batch were compared.

kinin-induced release of NO from five hearts was measured in the presence of hemoglobin by difference-spectrophotometry.

Results and Discussion

Endothelial cells

Stimulation of EC with BK (100 nM) resulted in the immediate release of nitric oxide which was paralleled by relaxant effects in the bioassay (Fig. 2). In all cases the NO release preceded the mechanical response and thus, constitutes an important argument for postulating NO˙ to be responsible for the observed vasorelaxation. The time interval required for endothelial cells to release NO after stimulation was 15 ± 1 s (n = 6). Interestingly, this interval was in the same range as the time required to increase cytosolic calcium in EC stimulated with BK (2), which is known to correlate with the production or release of EDRF (13). In our experiments bradykinin caused a threefold increase of endothelial NO release after the first challenge. Upon repetitive stimulation (BK, 100 nM, 3 min) endothelial NO formation rapidly decreased and this tachyphylaxis correlated well with the decrease in EDRF release (Fig. 2). Endothelial NO liberation was maximal at 1 ± 0.2 min after start of response, while relaxation maximum was reached at 3 ± 0.3 min. After cessation of BK infusion endothelial NO release reached basal levels in less than 1 min. Reversal of endothelium-derived relaxation was not attained before 10 min. Basal release of NO by endothelial cells was 16 pmol/min/ml column; which closely correlated with the basal release of EDRF.

In order to elucidate whether the concentrations of released NO are sufficient to explain the dilatory effect of EDRF we compared the maximal values of EDRF-mediated relaxation with the relaxation that

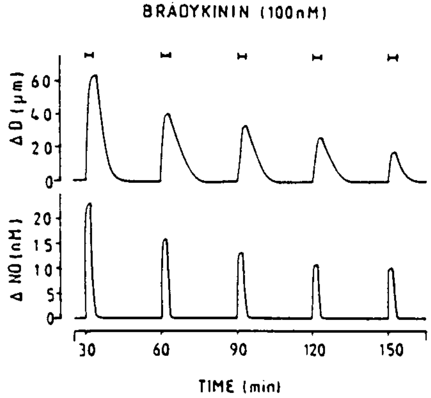

Fig. 2. Stimulation of EC with bradykinin (100 nM, 3 min, n = 5). Δμm: changes of diameter in the bioassay induced by release of EDRF, ΔNO: release of NO determined by difference-spectrophotometry. Values for Δμm and ΔNO were corrected for basal release of EDRF and NO.

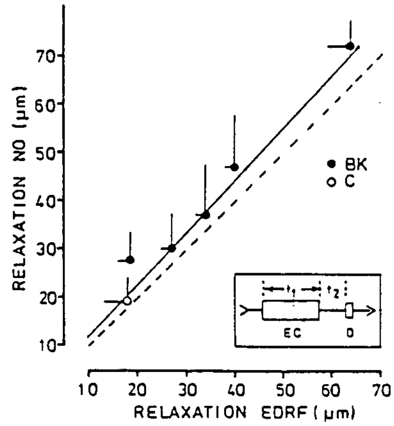

Fig. 3. Comparison of EDRF- and NO-induced relaxation. Data for maximal response elicited by bradykinin (BK, first to fifth stimulus) are from experiments shown in Fig. 2; C: relaxation induced by basal release of EDRF and NO. In the case of NO the assay permitted detection of total endothelial NO release, while EDRF was subject to degradation during passage to its site of detection in the bioassay. Therefore, NO concentrations measured in the photometric assay were corrected for half-life of NO and known transit time (t_1 and t_2, for details see (8)).

measured NO concentrations would have elicited in the bioassay. Since the photometric assay determines total cellular NO release not influenced by metabolism, respective values shown in Fig. 2 were first corrected for the half-life of NO and then converted into changes in vessel diameter using a dose-response curve for exogenously applied NO in the bioassay (for details see 8). From the data compiled in Fig. 3 it is evident that the correlation between EDRF- and NO-mediated relaxation is linear and is close to the line of identity. This finding clearly demonstrates that the observed smooth muscle relaxation was entirely due to the NO released by EC.

Isolated guinea pig hearts

Isolated hearts consistently released NO at a basal rate of 216 ± 36 pmol/min (n = 5). Bradykinin elicited an additional dose-dependent increase ranging from 70 ± 9 (BK: 5×10^{-10} M) to 300 ± 8 pmol/min (BK: 10^{-7} M). In contrast to cultured endothelial cells repetitive stimulation with bradykinin (100 nM, Fig. 3) did not cause a tachyphylaxis in the isolated heart for NO release and coronary vasodilation. Consistent with the observed NO release is the finding that hemoglobin (4 μM) significantly shifted the dose response curve for the bradykinin-induced decrease of coronary perfusion pressure to the right (data not shown). Furthermore, the onset of NO release preceded the onset of the hemodynamic response consistently by 2 s. This interval was in the same range as the response time of coronary vascular smooth muscle when NO was applied intracor-

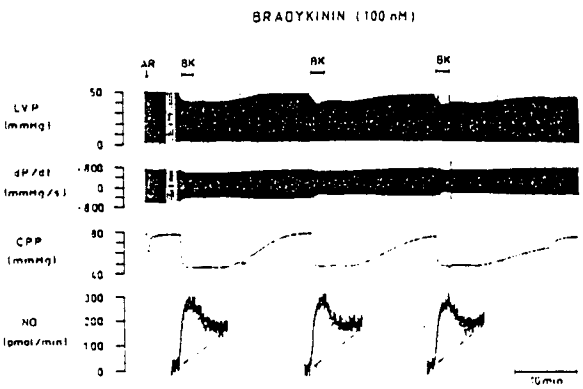

Fig. 4. Representative tracing of the effect of bradykinin (Bk, 100 nM, 2 min, n = 5) on hemodynamic parameters of the isolated guinea pig heart (coronary flow: 10 ml/min) and the release of NO into the coronary effluent perfusate. LVP: left ventricular pressure, CPP: coronary perfusion pressure, ∅NO: release of nitric oxide in addition to basal release, AR: bolus injection of adenosine (50 µl, 50 µM).

onarily. The amount of released NO was within the vasodilatory range of intracoronarily applied NO (9).

Comparison of NO release from isolated hearts and cultured macrovascular endothelial cells

Comparing the release of NO under in vivo conditions (isolated heart) with cultured macrovascular endothelial cells reveals the following interesting features:

1) Bradykinin at a maximally effective concentration (100 nM) increased basal NO release in both experimental models two to three fold. Since isolated hearts as well as cultured cells were perfused at a constant volume, this increase cannot be attributed to a washout phenomen.

2) Onset of NO release always preceded the start of vasodilation. The interval between these two parameters was 45 s in the case of cultured EC and 2 s in the isolated heart. This response time of the vascular smooth muscle was in both experimental preparations identical for endogenously released and exogenously applied NO.

3) The interval required for bradykinin to increase endothelial NO release via the receptor mediated pathway is much shorter in the isolated heart (< 2 s) than in cultured macrovascular endothelial cells (< 15 s). This may be due to differences in the species (hog vs guinea pig) and in the origin of the endothelial cells (micro- vs macrovascular EC). It is also conceivable that the culture condition as such may alter the responsiveness.

4) In contrast to the conditions of the isolated heart the bradykinin receptor in cultured endothelial cells is subject to tachyphylaxis upon repetitive stimulation with bradykinin. A possible explanation for this finding could be, that the bradykinin receptor itself (1) or intracellular mechanisms participating in the signal-transduction pathway for NO release are altered in cultured endothelial cells. Consistent with this interpretation is our observation that calcium ionophore (10^{-6} M) still elicited a maximal NO release in bradykinin-desensitized cultured endothelial cells (data not shown).

Conclusions

Evidence is provided that the free nitric oxide radical is released from cultured macrovascular endothelial cells as well as from the isolated guinea pig heart under basal conditions. The amount of released NO from cultured EC can completely explain the vasodilatory effect of EDRF; no additional EDRFs need to be postulated in this model. Bradykinin dose-dependently decreases coronary perfusion pressure and increases the release of NO into the coronary effluent perfusate. Amount and time-course of released NO suggest that the NO formed within the heart can influence the tone of coronary resistance vessels and is thus involved in the regulation of coronary vascular resistance.

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