Vol. 154, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1988 Pages 236-244

QUANTITATIVE AND KINETIC CHARACTERIZATION OF NITRIC OXIDE AND EDRF RELEASED FROM CULTURED ENDOTHELIAL CELLS

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Received June 1, 1988

Endothelial cells (EC) contribute to the control of local vascular diameter by formation of an endothelium derived relaxant factor (EDRF) (1). Whether nitric oxide (NO) is identical with (EDRF) or might represent only one species of several EDRFs has not been decided as yet (2-5). Therefore, we have directly compared in cultured EC the kinetics of NO formation determined in a photometric assay with the vasodilatory effect of EDRF and NO in a bioassay. Basal release of NO was 16,4 pmol/min/ml packed EC column. After stimulation with bradykinin (BK) and ATP onset of endothelial NO release and maximal response preceded the EDRF-mediated relaxation. Concentrations of NO formed by stimulated EC were quantitatively sufficient to fully explain the smooth muscle relaxation determined in the bioassay. Our data provide convincing evidence that under basal, BK and ATP-stimulated conditions 1. endothelial cells release nitric oxide as free radical, 2. nitric oxide is solely responsible for the vasodilatory properties of EDRF.

EDRF released by endothelial cells mediates the vasodilatory effect of various substances including bradykinin and ATP which envolves activation of guanylate cyclase in the vascular smooth muscle (VSM) (6). Previously it was shown that EDRF formation was associated with endothelial release of nitric oxide (2). Based on the similarities of NO and EDRF to cause relaxation and cGMP elevation in the VSM it was suggested that NO might be identical with EDRF (2,7). In contrast, recently reported results described different biological and chemical properties of EDRF and NO (3,8)

0006-291X/88 $1.50

and aside of NO substances such as ammonia or hydroxyl radicals were ascribed to EDRF (4,5). Moreover, it is unknown whether EC release nitric oxide as a free radical or an endogenous precursor which subsequently gives rise to NO formation in the vascular smooth muscle.

Because of the instability of NO and the lack of a method to directly measure NO at its cellular site of formation, no data are available comparing the kinetics of EDRF and NO formation. This, however, is an important prerequisite for establishing a cause and effect relationship between NO and vascular relaxation. Furthermore, basal release of EDRF from unstimulated EC has been shown to modulate vascular tone (9-11) but no information is available whether this is caused by a basal release of NO. Therefore, we elaborated a sensitive method, which allowed to directly compare the kinetics and quantities of NO formed by cultured EC with the vasodilatory effect of EDRF and NO in a bioassay.

MATERIALS AND METHODS

Endothelial cells . EC were isolated from porcine thoracic aorta and cultured in M199 medium plus 20 % foetal calf serum. Purity of culture was determined by immunological fluorescent staining of factor VIII related antigen, phase-contrast- and scanning electron microscopy. 8-15 days after isolation cells were grown on microcarrier beads (Biosiolon, Nunc, $5\text{-}7 \times 10^6$ cells/g bead)(12), packed into a column and perfused at 2 ml/min with a HEPES-buffered Krebs-Ringer solution containing (mM) NaCl 140, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.03, NaH_2PO_4 0.42, glucose 5.0, pyruvate 2.0, HEPES 10.0 (pH 7.4, equilibrated with room air at a temperature of 37°C). Medium was additionally supplemented with 10 µM indomethacine. Parallel measurement of EDRF and NO release in two columns 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. Packed bead volume of the column used for the bioassay was always 1 ml. Bead volume of the column used for NO measurement varied between 2 - 4 ml. A linear relationship exists between concentration of EC derived NO and column volume. Therefore, all results are normalized to 1 ml packed bead volume which contained 0,6 g beads and a void volume of 381 ± 16 µl(n=8).

Photometric assay . For the quantification of EC-derived NO a difference-spectrophotometric method was used, which is based on the rapid oxidation of oxyhemoglobin to methemoglobin by nitric oxide (13): $Hb(II)O_2 + NO \rightarrow Met(III)Hb + NO_3^-$. Previously described specificity (13,14) was confirmed by chemiluminescence (15). We determined the time for complete reaction of HbO_2 with

NO to be less than 100 ms (n=6) using a stopped-flow technique. HbO_2 traps the entire formed NO and prevents its further degradation; hence it permits quantification of the actual rate of NO formation by EC. Measurement of the extinction difference (λ_1 =401 nm, λ_2 =411 nm) in a flow-through cell permitted continuous assay of EC-derived NO. NO concentrations were calculated from the determined extinction coefficient ($\Sigma_{401-411\ nm}$: 38 mM^{-1} cm^{-1}) and validated by infusion of aqueous NO standards. Preparation of HbO_2 has been described in detail previously (12). Briefly, bovine hemoglobin was dissolved in water (pH 7), equilibrated with oxygen, reduced with a molar excess of natriumdithionite and purified by gelchromatography (Sephadex G 25, Pharmacia). Purity was controlled spectrophotometrically and by PAGE.

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. Removal of endothelium by deoxycholic acid (0,75%, 15sec) was verified by transmission and scanning electron microscopy and lack of responsiveness to acetylcholine (1µM). Vessel diameter was measured by a pair of piezoelectrical crystals (4MHz, detection limit of diameter changes: 2 µm). Norepinephrine (0,2 µM) reduced vessel diameter by an average of 213 ± 8 µm from a control value of 1,138 ± 0,038 mm (n=14) .

Nitric oxide solutions. Standards of aqueous NO solutions were prepared by saturation of argon-degassed and 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, which is described in detail elsewhere (15).

Drugs, statistical analysis. The following substances were used: ATP, bradykinin, indomethacine, and bovine hemoglobin (Sigma); acetylcholin (Dispersa); superoxide dismutase (EC 1.15.1.1, Serva); adenosine (Boehringer); natriumdithionite (Merck). Results are reported as mean ± SEM.

RESULTS AND DISCUSSION

Sensitivity of vascular smooth muscle for nitric oxide. Administration of exogenous applied NO to the bioassay resulted in the demonstrated dose response curve (Fig.1). The threshold concentration for NO was 1 nM, EC_{50} = 5nM. The vasodilatory effect of NO (5nM) was independent of the precontraction of the vessel elicited by norepinephrine in the range of 0.1 - 1 µM (n=3). The mean response time of the vascular smooth muscle, defined as the interval between infused NO reaching the vessel and onset of vasodilation, was determined to be 45 ± 5 sec (n=22).

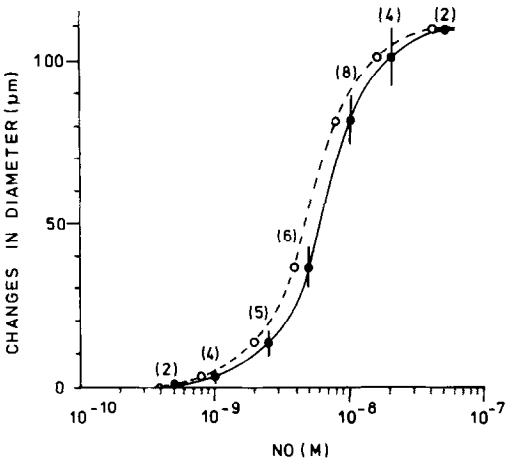

Fig. 1 Dose response curve of the vasodilatory effect of exogenously applied NO on endothelium-denuded, precontracted isolated rat aortas . Changes in diameter were recorded with a pair of piezoelectrical crystals. Transit time from site of infusion of NO standards to the piezoelectrical crystals was 1,8 s. True sensitivity of the vessel to NO (dashed line) was calculated from measured values (solid line) corrected for transit time and known half-life of NO. (numbers in parenthesis: number of vessels tested)

Half-life of nitric oxide. Half-life of NO under the experimental conditions used in this study was determined by infusion of NO into a tubing system. Sites of infusion were seperated from the site of HbO_2 infusion (4µM) by a transit time of 0-15 s. The difference-spectrophotometric method has the advantage of a brief response time (<100 ms) and permitted the first precise estimate of NO half-life by a chemical method: 5,6 s (pseudo-first order, constant of evasion $k=0,124 \pm 0,001\ sec^{-1}$, n=10). Previously reported half-life determined for EDRF and NO using different assays ranged from 6-30 s (2,10,16).

Basal release of nitric oxide and EDRF: HbO_2 exhibits a high affinity for NO and is known to be a potent inhibitor of EDRF (7, 11). Superfusion of EC with 4 µM HbO_2 unmasked basal EDRF release which resulted in a further contraction of the vessel by 18 ± 5µm (n=5). This is consistent with previously reported results, which demonstrated basal release of EDRF by infusion of superoxide dismutase (SOD) (9). The concentration of endothelial NO formed

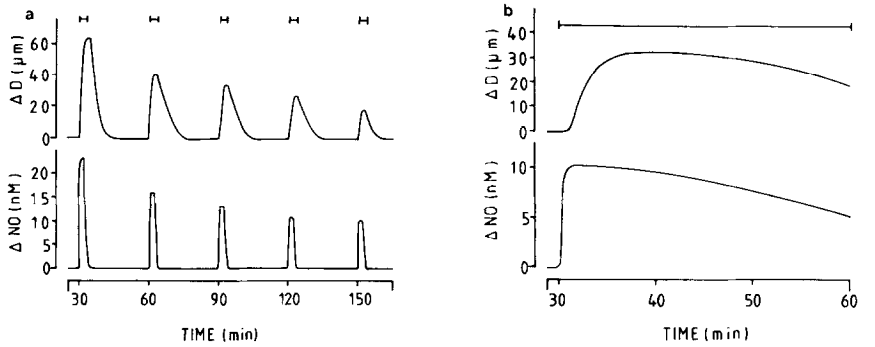

Fig. 2 Cultured EC grown on microcarriers beads were perfused in a column (2 ml/min) and stimulated with bradykinin (Fig. 2a: BK, 100 nM, 3 min, n=5) and adenosine 5'-triphosphate (Fig. 2b: ATP, 100 μM, 30 min, n=3). Release of EDRF was continously determined in the bioassay. Changes in NO release were 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.

under basal conditions was determined to be 8.2 ± 1.4 nM (n=10), which corresponds to a rate of NO release of 16.4 pmol/ min for 1 ml packed column. Superfusion of EC column with the hemoglobin containing perfusion medium plus SOD (100 U/ml, n=3) did not alter endothelial NO release, neither under basal nor stimulated conditions. Hence, the NO scavenging ability of HbO_2 is much faster than the O_2^- dismutation by SOD. Moreover, in the presence of HbO_2 SOD does not interfere with the liberation of endothelial NO.

Bradykinin induced release of nitric oxide and EDRF. Stimulation of EC with BK (100 nM) and ATP (100 μM) resulted in the immediate release of nitric oxide which was paralleled by relaxant effects in the bioassay (Fig. 2) . In all cases did the NO release precede the mechanical response and thus constitutes an important argument for postulating NO to be responsible for the observed vasorelaxation. The interval required for endothelial cells to release NO after stimulation was 15 ± 1 s (n=6) for both ATP and BK. Interestingly, this interval was in the same range as the time required to increase cytosolic calcium in EC

stimulated with BK (17), which is known to correlate with the production or release of EDRF (18). In our experiments bradykinin caused a 3-fold increase of endothelial NO release after the first challenge. Upon repetitive stimulation (BK, 100nM, 3 min) endothelial NO formation rapidly decreased and this tachyphylaxis correlated well with the decrease in EDRF release (Fig. 2). After first stimulation with BK changes in vessel diameter were 64 ± 5 µm and NO concentration increased by 23.1 ± 2.6 nM corresponding to 46.2 pmol NO/min/ ml packed bead volume. 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 endothelial derived relaxation was not attained before 10 min.

ATP induced release of nitric oxide and EDRF. Similiar results as with BK were obtained with ATP (100µM, n=3) . Again, NO release preceded smooth muscle relaxation. Maximum of NO liberation (10.1 ± 2.8 nM) was reached after 1.3 ± 0.2 min, maximum of vasodilation (32 ± 5 µm) not before 9 min. In contrast to the marked tachyphylaxis observed with BK, the ATP induced NO release only slowly declined with time (after 30 min: 50 ± 7 % of the maximum). Repetitive stimulation of EC with ATP (100 µM, 4 stimuli of 10 min, stimulation free interval : 30 min) did not significantly change the release of NO (data not shown). This is in accordance with previously reported results (19), showing unaltered EDRF release after repetitive stimulation with ATP. EC of higher passages (>30 days) lost their ability to release NO or EDRF when stimulated with BK but not with ATP. Furthermore, adenosine (10 µM) did not elicit any NO release (n=3).

Quantitative comparison of EDRF and Nitric Oxide. In order to elucidate whether the concentrations of released NO are suffi-

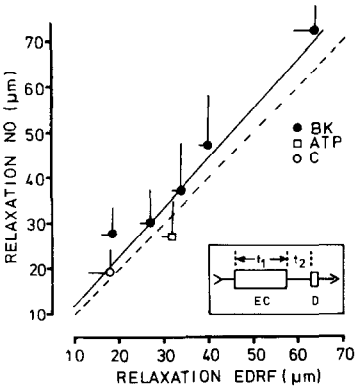

Fig. 3 Comparison of EDRF and NO - induced relaxation. Data for maximal response elicited by bradykinin (BK, first to fifth stimulus) and adenosine 5'-triphosphate (ATP) are from experiments shown in Fig. 2; C: relaxation induced by basal release of EDRF and NO. In the case of NO the assay permitted 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. Transit time (t_1) over the column packed with 1 ml EC covered beads was 11,4 s (see inset); transit time (t_2) from the end of the column to the detecting system (D) was 4,2 s. Assuming NO release to follow zero order kinetic, the correction factor (F) can be described by the equation: $F = \frac{k_2 \cdot t_1 \cdot e^{k_2 \cdot t_2}}{1 - e^{-k_2 \cdot t_2}}$ where k_2 is the constant of evasion for NO (see appendix). The corrected values for NO were converted to changes in diameter using the dose-response curve for NO (Fig. 1). Mean values are expressed as x ± SEM. Line of identity (dashed line), linear regression analysis (solid line) : y = 1,09 x + 0,74; r = 0,965; $p \leq 0,001$; n = 7.

cient to explain the dilatory effect of EDRF we compared the maximal values of EDRF-mediated relaxation with the relaxation that 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 the dose-response curve shown in Fig. 1. From the compiled data in Fig. 3 it is evident that the correlation between EDRF- and NO-mediated relaxation is linear and falls 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.

In conclusion our kinetic and quantitative data demonstrate that the NO release of cultured endothelial cells can completely

explain the vasodilatory effect of EDRF. No additional EDRFs need to be postulated in this experimental model. Furthermore, NO is released as a free radical. The photometric assay of NO should provide a powerful tool for the further elucidation of the mechanisms of endothelial NO-production and release.

ACKNOWLEDGEMENTS. We wish to thank K.Kroll, PhD., for advice with the bioassay, Dr. G. Steger for help with the photometric stopped-flow technique, Dr. A. Deussen and Mr.W. Gunder for helpful discussion and Mrs. C. Kirberich for skilfull technical assistence. M.Feelisch is a recipient of a SKD-scholarship

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APPENDIX. Formation of NO was described as $\frac{dc}{dt} = k_1$ and its decay as $\frac{dc}{dt} = -k_2 C$. A steady state of both processes was achieved under basal conditions as well as upon each EC

stimulation. Integration of both processes $\left(\frac{dc}{dt} = k_1 - k_2 \cdot c\right)$ reveal a concentration of NO at the end of the column as $c_1 = \frac{k_1}{k_2}\,1 - e^{-k_2 \cdot t_1}$ and at the detector as $C_2 = C_1 \cdot e^{-k_2 \cdot t_2}$. The quotient of C_2 (C_2=concentration of NO that would have reached the detector in the absence of HbO_2) to C_p ($C_p = k_1 \cdot t_1$, concentration of NO, respective MetHb, in the presence of HbO_2) describes the factor F (see Fig.3).