

Regulation of eNOS expression in HCAEC cell line treated with opioids and proinflammatory cytokines

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Abstract

Background: Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) plays a crucial role in vascular function and homeostasis. eNOS activity maintains normal vascular tone, regulates leukocyte-endothelial cell interactions, inhibits platelet aggregation, limits smooth muscle cell proliferation and influences cardiac myocyte contractility. The loss of endothelium-derived NO has been shown to result in serious cardiovascular abnormalities, which may indicate eNOS involvement in the origin/development of cardiovascular disorders.

Aim: Evaluation of eNOS mRNA level in the endothelium of human coronary arteries upon opioids treatment (mediators of ischaemic preconditioning) and after incubation with proinflammatory cytokines (stress stimuli).

Methods: Different concentrations of β -endorphin, endomorphin-1 and endomorphin-2 (alone or in combination with the opioid receptor blocker naloxone) as well as different concentrations of cytokines alone (IL-1 β , TNF- α) or in combination were applied to *in vitro* cultured human coronary artery endothelial cells (HCAEC). After 24 hrs incubation, the cells were harvested, mRNA extracted and relative quantification of eNOS mRNA was conducted using real-time PCR.

Results: Opioid treatment altered eNOS expression; however, none of the changes reached a statistically significant level. As for proinflammatory cytokines, both TNF- α and IL-1 β substantially down-regulated the eNOS mRNA level ($p < 0.05$). When applied in combination, these cytokines lowered eNOS mRNA even further ($p < 0.05$). The effect was independent of the cytokine combination used.

Conclusions: It was demonstrated that proinflammatory cytokines exert a substantial and statistically significant negative effect on eNOS mRNA level in human coronary artery endothelial cells in *in vitro* culture. Unfortunately, we were unable to demonstrate significant changes within the eNOS mRNA pool upon opioid treatment. These results indicate that opioids (basal release) do not affect eNOS expression in normal *in vitro* culture conditions but might do so upon stress stimuli.

Key words: eNOS, opioids, proinflammatory cytokines, cardiovascular disease

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Introduction

Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) plays a crucial role in vascular function and homeostasis. eNOS activity maintains normal vascular tone, regulates leukocyte-endothelial cell interactions, inhibits platelet aggregation, limits smooth muscle cell proliferation [1, 2] and influences cardiac myocyte contractility through L-type calcium

channel inhibition [1]. Its presence was originally found in endothelial cells; later wide distribution of eNOS was also demonstrated in the heart within cardiac myocytes, cardiac endothelium and vascular endothelial cells of the coronary arteries [1].

The loss of endothelium-derived NO results in serious vascular abnormalities including vasoconstriction, smooth muscle cell proliferation and adhesion of blood

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elements to the vessel wall [3]. Therefore, decreased eNOS expression may contribute to the development of cardiovascular diseases including acute myocardial infarction (MI). Moreover, abnormalities in eNOS-mediated vasorelaxation have been observed in patients after infarction suffering from chronic heart failure [4]. The fact that eNOS overexpression has been shown to attenuate heart failure in animal models [2, 4] strongly suggests that it has an important role in the origin of cardiovascular disorders.

Acute MI is associated with substantially elevated levels of proinflammatory cytokines such as TNF- α and IL-1 β [3]. High levels of these cytokines have also been shown during congestive heart failure and other cardiovascular diseases, which suggests their further involvement in the progression of heart failure [5]. Since it is well documented that NO-mediated vasorelaxation is seriously impaired in MI patients, we decided to verify the hypothesis that the decrease in endothelium-produced NO is a result of cytokine-mediated downregulation of eNOS.

The endogenous opioid system is activated during inflammation as a physiological feedback mechanism to attenuate inflammatory pain [6]. It is well known that opioids exert important cardiovascular effects including regulation of regional blood flow and blood pressure, and modulation of vascular smooth muscle tone [7]. Only recently, opioids turned out to be the key mediators of ischaemic preconditioning (IPC), a phenomenon in which brief episodes of ischaemia and reperfusion limit myocardial damage induced by prolonged ischaemic insult [8]. The protective effect of opioids is sustained up to 72 hrs, indicating the involvement of *de novo* protein synthesis. Considering the physiological role of eNOS, it seems reasonable to hypothesise that its activation during MI would be beneficial. To confirm this assumption, we decided to determine whether opioid peptides, the key mediators of the IPC process, may influence eNOS expression in the endothelium of human coronary arteries.

Methods

Cell culture

Human coronary artery endothelial cells (HCAEC) purchased from Clonetics (San Diego, CA, USA) were grown in 25 cm² flasks in the EGM-2-MV[®] medium (Clonetics) under conditions recommended by the manufacturer. Briefly, the cultures were incubated in a 5% CO₂ atmosphere at 37°C and passaged when reaching confluence. In order to diminish any changes in cell physiology caused by prolonged *in vitro* culture,

the cells were only used within three to five passages. All experiments were performed at cell confluence.

Opioid treatment

HCAEC were incubated for 24 hrs in 5 ml aliquots of the medium under the following conditions: 1. β -endorphin (10 nM), 2. β -endorphin (10 nM) and opioid receptor blocker naloxone (1 μ M), 3. β -endorphin (1 μ M), 4. β -endorphin (1 μ M) and naloxone (1 μ M), 5. endomorphin-1 (1 μ M), 6. endomorphin-1 (1 μ M) and naloxone (1 μ M), 7. endomorphin-1 (10 μ M), 8. endomorphin-1 (10 μ M) and naloxone (1 μ M), 9. endomorphin-2 (1 μ M), 10. endomorphin-2 (1 μ M) and naloxone (1 μ M), 11. endomorphin-2 (10 μ M), 12. endomorphin-2 (10 μ M) and naloxone (1 μ M). The cells treated with growth medium alone served as controls. In order to check whether the changes in eNOS expression were due to the opioid treatment but not to the fluctuations in constitutive level of eNOS mRNA, each experiment consisted of two control sample flasks and every opioid treatment was carried out four times.

Cytokine treatment

HCAEC were incubated in the presence of proinflammatory cytokines for 24 hrs. The medium contained the following components: 1. IL-1 β (2 ng/ml), 2. IL-1 β (20 ng/ml), 3. TNF- α (2.5 ng/ml), 4. TNF- α (25 ng/ml), 5. IL-1 β (2 ng/ml) and TNF- α (2.5 ng/ml), 6. IL-1 β (20 ng/ml) and TNF- α (25 ng/ml), 7. IL-1 β (2 ng/ml) and TNF- α (25 ng/ml), 8. IL-1 β (20 ng/ml) and TNF- α (2.5 ng/ml). Cell aliquots incubated in growth medium alone served as controls. Each treatment was performed three times.

Relative quantification of eNOS mRNA

In order to carry out eNOS mRNA quantifications, the cells were trypsinised, centrifuged and counted. Total RNA from approximately 2–6 \times 10⁵ cells was obtained using the GTC/phenol extraction method. RNA concentration was determined spectrophotometrically at 260 nm. RNA integrity was evaluated by 1% agarose gel electrophoresis. 3 μ g of total RNA was subjected to reverse transcription using MMLV reverse transcriptase (Ambion). PCR primers were taken from PrimerBank (ID 10835161a3 and 4501885a1 for eNOS and β -actin, respectively). Relative quantification of eNOS mRNA level was assessed by real-time PCR using the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, USA) in the presence of SYBR green. Primer specificity and PCR product verification was achieved by the generation of a melting curve followed by 1% agarose gel electrophoresis. The level of eNOS mRNA was normalised against endogenous control (β -actin).

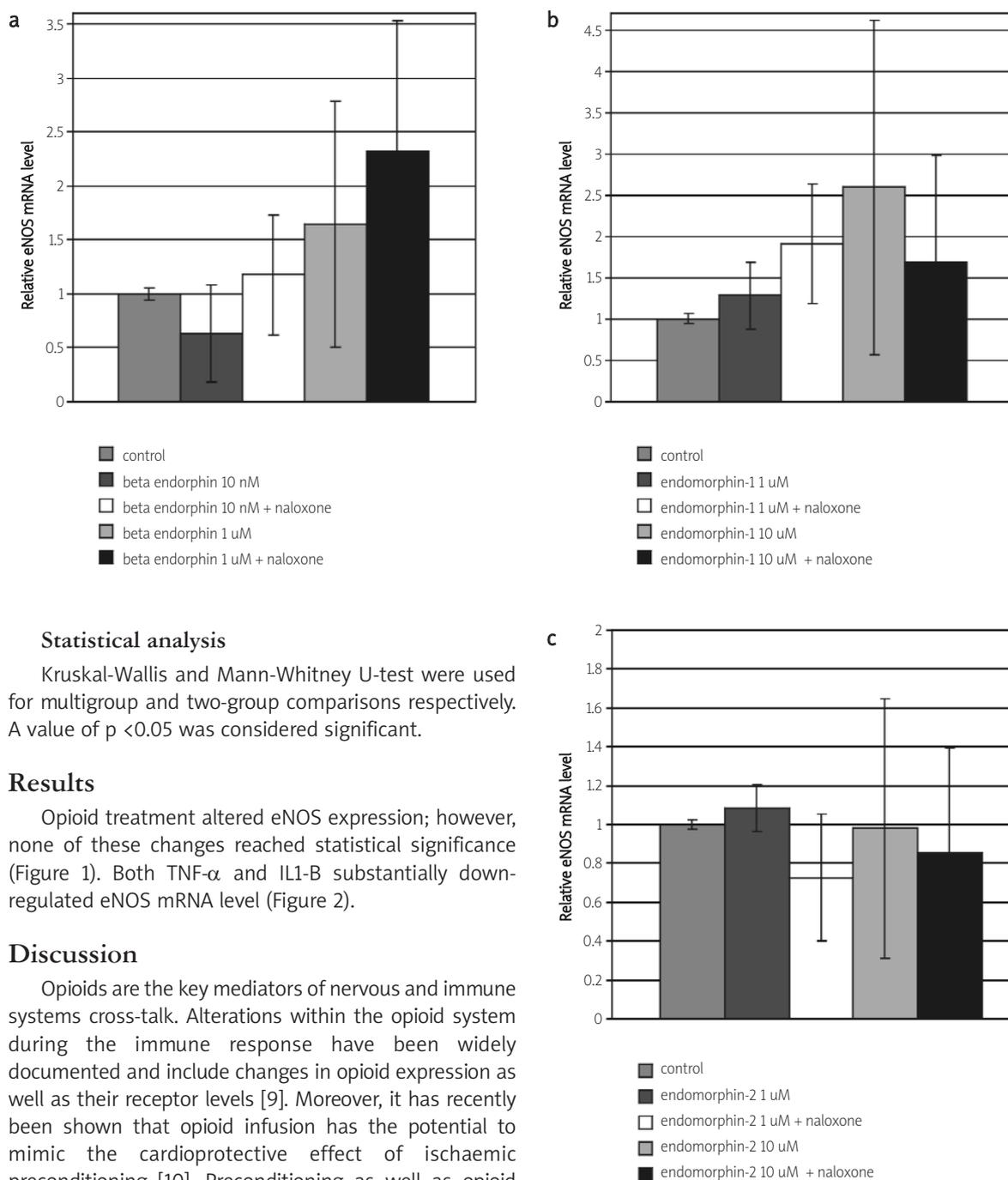


Figure 1a-c. Relative eNOS mRNA content upon treatment with: a) β -endorphin, b) endomorphin-1, c) endomorphin-2

Statistical analysis

Kruskal-Wallis and Mann-Whitney U-test were used for multigroup and two-group comparisons respectively. A value of $p < 0.05$ was considered significant.

Results

Opioid treatment altered eNOS expression; however, none of these changes reached statistical significance (Figure 1). Both TNF- α and IL1-B substantially down-regulated eNOS mRNA level (Figure 2).

Discussion

Opioids are the key mediators of nervous and immune systems cross-talk. Alterations within the opioid system during the immune response have been widely documented and include changes in opioid expression as well as their receptor levels [9]. Moreover, it has recently been shown that opioid infusion has the potential to mimic the cardioprotective effect of ischaemic preconditioning [10]. Preconditioning as well as opioid administration reduces infarct size in experimental animals and limits cell death in isolated cardiomyocytes [11, 12]. Opioid peptides are released upon stress stimuli (e.g. MI) and it is believed that they act as compensatory mediators to diminish the deleterious effects of an ongoing inflammatory reaction [12]. As for now, the precise signalling pathway involved in opioid-induced cardioprotection remains unclear and is still under investigation. The time course (12–42 hrs) and duration of delayed IPC strongly implicates *de novo* synthesis of

cardioprotective proteins. The physiological role of eNOS puts this enzyme among the strong candidates for mediators of cardioprotection. Compelling evidence for its beneficial role in cardiovascular disorders was provided by

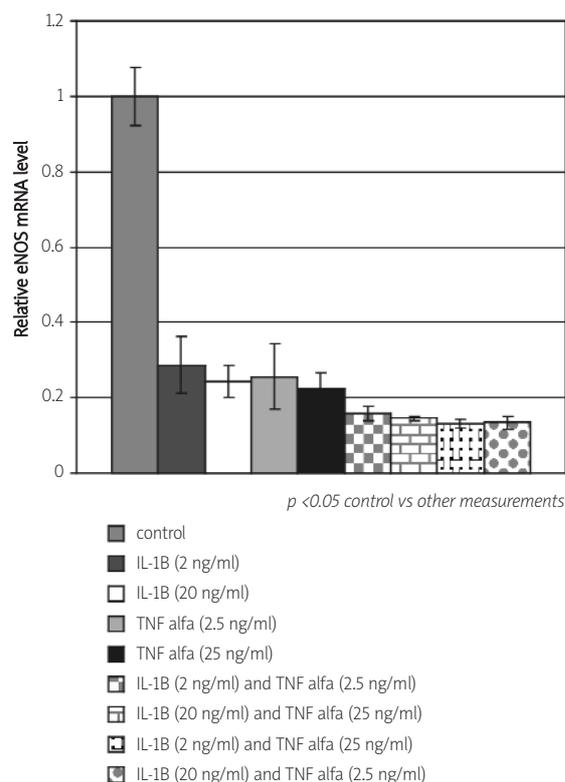


Figure 2. Relative mRNA eNOS content upon proinflammatory cytokine treatment

generation of transgenic mice overexpressing human eNOS [2]. The protective effects of eNOS may include blunting of the vasoconstrictor response, remodelling and formation of new vessels within the scar, and scavenging superoxide and other reactive oxygen species released under stress [2]. Moreover, NO derived from eNOS has been shown to have the potential to diminish a proinflammatory event. The mode of action involves inhibition of NF- κ B, which is a prerequisite for cytokine activation [13].

It is well documented that the heart is an abundant source of opioid precursors [10], which are released from cardiomyocytes during stress. The mode of action may involve either the autocrine process in which peptides interact directly with the relevant myocyte receptors, or paracrine interaction with receptors on other cell types in the heart, such as endothelial cells.

We have demonstrated (Figure 1) that opioid treatment altered eNOS expression; however, none of the changes reached a statistically significant level ($p > 0.05$). Although the experimental protocol was validated thoroughly [15], we observed considerable variations among biological replicates. The factors accounting for this inconsistency are unclear.

Both TNF- α and IL-1 β substantially downregulated eNOS mRNA level ($p < 0.05$) (Figure 2). There was no statistically significant difference between TNF- α -induced and IL-1 β -induced downregulation of eNOS. Low doses of both cytokines (2 ng/ml and 2.5 ng/ml for IL-1 β and TNF- α , respectively) caused a considerable decline in eNOS mRNA, which was not significantly deepened by ten-fold higher cytokine concentrations. Cytokine combinations were much more potent in eNOS mRNA downregulation than each of the cytokines applied separately ($p < 0.05$). The effect was independent of the cytokine combination used.

Local and systemic elevation of proinflammatory cytokines is associated with cardiovascular disease [3] and may lead to severe endothelial dysfunction [16]. Substantial cytokine upregulation has been shown to occur during acute MI as a natural consequence of stress [5]. The degree of cytokine-induced downregulation of eNOS may correspond to the severity of MI as well as to further development of the heart failure process. Endothelial NOS malfunctioning results in vasoconstriction, smooth muscle cell proliferation and adhesion of blood elements such as leukocytes and platelets to the vessel wall [3]. These abnormalities may considerably affect the degree of heart muscle destruction during MI.

The statistical insignificance of our results obtained in the experiments with opioids does not exclude eNOS participation in the preconditioning phenomenon. Stress induced due to an inflammatory reaction causes a substantial increase not only in opioid transmitters but most of all in proinflammatory cytokines. Thus, stress stimuli (e.g. proinflammatory cytokines release itself) might be a prerequisite for opioids to manifest their effect on eNOS expression. The inflammatory reaction is associated with the alteration within opioid receptor expression, and in this regard cytokine pretreatment may be essential for the expression of relevant opioid receptors on endothelial cells. On the other hand, the opioid system may be part of a feedback mechanism in which opioids abolish the negative cytokine effect on eNOS. This could indicate an important mechanism of cardioprotection which remains to be elucidated.

Conclusions

In our study we demonstrated that pro-inflammatory cytokines exert a substantial and significant negative effect on eNOS mRNA level in human coronary artery endothelial cells in *in vitro* culture. Unfortunately, we were unable to demonstrate statistically relevant changes within the eNOS mRNA pool upon opioid treatment. These results indicate that opioids (basal release) do not affect eNOS expression in normal *in vitro* culture conditions but might do so upon stress stimuli. This hypothesis remains to be verified.

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Regulacja poziomu eNOS w komórkach śródbłonka HCAEC pod wpływem opioidów prozapalnych

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Streszczenie

Uzasadnienie: Tlenek azotu (NO) generowany przez śródbłonkową syntazę tlenku azotu (eNOS) pełni podstawową rolę w utrzymywaniu homeostazy naczyniowej. Aktywność eNOS zapewnia prawidłowe napięcie naczyniowe, reguluje interakcje międzykomórkowe leukocyty – śródbłonek, hamuje agregację płytek, ogranicza proliferację komórek mięśni gładkich oraz wpływa na kurczliwość kardiomiocytów. Znaczny spadek poziomu NO w obrębie śródbłonka prowadzi do poważnych zaburzeń naczyniowych, co sugeruje, że zaburzenia ekspresji eNOS mogą w istotny sposób przyczyniać się do rozwoju chorób układu sercowo-naczyniowego.

Cel: Ilościowa analiza poziomu mRNA dla eNOS w śródbłonku ludzkiej tętnicy wieńcowej (HCAEC) po inkubacji komórek w medium zawierającym opioidy (mediatory hartowania przez niedokrwienie) oraz cytokiny prozapalne (imitacja warunków stresowych).

Metody: Hodowla *in vitro* komórek linii HCAEC w obecności różnych stężeń β -endorfiny, endomorfiny 1 i 2 stosowanych odrębnie lub w połączeniu z niespecyficznym inhibitorem receptorów opioidowych – naloksonem oraz w obecności różnych stężeń cytokin prozapalnych (IL-1 β lub TNF- α) stosowanych osobno lub w kombinacji. Po 24 godzinach inkubacji, komórki odtrawiano, ekstrahowano RNA i przeprowadzono ilościową analizę mRNA dla eNOS metodą PCR w czasie rzeczywistym.

Wyniki: W cyklu prowadzonych doświadczeń wykazano wpływ opioidów na ekspresję eNOS, jakkolwiek żadna z zaobserwowanych zmian nie osiągnęła poziomu istotności statystycznej ($p > 0,05$). Dla obu cytokin prozapalnych (TNF- α i IL-1 β) zaobserwowano statystycznie znamienne spadki poziomu mRNA dla eNOS, który ulegał dalszemu obniżeniu po zastosowaniu obu cytokin równocześnie ($p < 0,05$). Efekt ten był niezależny od zastosowanej kombinacji.

Wnioski: Wykazano, że hodowla ludzkich komórek śródbłonka tętnicy wieńcowej w obecności cytokin prozapalnych prowadzi do znacznego obniżenia poziomu mRNA dla eNOS ($p < 0,05$). Nie udało się zaobserwować statystycznie znamiennych różnic w ekspresji eNOS pod wpływem opioidów. Uzyskane wyniki wskazują, że opioidy nie wywierają istotnego wpływu na poziom mRNA dla eNOS w standardowych warunkach hodowli *in vitro*, natomiast nie można wykluczyć, że mogą one modulować ekspresję eNOS podczas ostrej reakcji zapalnej.

Słowa kluczowe: eNOS, opioidy, cytokiny pro-zapalne, choroba naczyniowo-sercowa

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