Electrochemical sensing of angiogenin induced endothelial nitric oxide synthase activity

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Abstract: Angiogenesis, formation of new blood vessels, is a complex but critical phenomenon. In particular, it is regulated by different angiogenic factors. Nitric oxide (NO) is also a very well known biological mediator involved in vascular physiology. This study focuses on relationships between the effect of angiogenin, a major angiogenic factor, and extracellular NO release. NO concentration was sensed electrochemically using a fibronectin coated multiple microelectrode array. Angiogenin was shown to increase NO levels, thus triggering nitric oxide synthase (NOS) activity. Angiogenin reactive pathway being very complex, we have used various selective inhibitors of angiogenin to investigate the mechanism leading to NO production. Neomycin, an antibiotic blocking nuclear translocation, inhibited angiogenin effect on NOS. This result demonstrates that angiogenin activates NOS by interacting with the cell nucleus.

Keywords: angiogenesis, electrochemical sensing, nitric oxide synthase, biomeasurements, stem cells, angiogenin

Introduction

Angiogenesis, formation of new blood vessels, is critical factor in much pathology like chronic wounds (Harding et al., 2002) and rheumatoid arthritis (Walsh, 1999). In particular, it has been proven that tumours release angiogenic factors in order to support their growth, carcinogenesis being strongly dependent on blood supply (Carmeliet et al., 2000). However, angiogenesis is a rather complex biological phenomenon that is dependent on the extra cellular matrix (Clark, 1995), mechanical or shear stress (Baum et al., 2004) and biochemical factors. For example, some growth factors like vascular endothelial growth factors are known to promote endothelial growth and angiogenesis (Ferrara, 2001). This biological phenomenon involves the activation of blood vessel cells to an altered state as a response to a complex balance of competing stimuli. Investigating its biochemistry therefore seems the most obvious way to understand and modulate angiogenesis.

In this study, we are focusing on an angiogenic protein, angiogenin. Angiogenin is a 123 amino acids long single-chain protein of about 14,400 kDa (Gao and Xu, 2008). This molecule is a requirement for cell proliferation, even for angiogenesis induced by other angiogenic factors, like fibroblast growth factors or endothelial growth factors (Kishimoto et al., 2005). In particular, it has been reported that nuclear translocation of angiogenin, e.g. angiogenin entering cell nucleus, is a critical step for the biochemical cascade triggering angiogenesis (Kishimoto et al., 2005). Furthermore, angiogenin is known to have several effects on cells (Gao and Xu, 2008):

(i) it can bind to the actin receptors and cleave the extracellular matrix, thus allowing cell migration (Hu et al., 1994),
(ii) it has an RNase activity (Russo et al., 1994),
(iii) it can bind to cell membrane receptors and trigger an

intracellular biochemical cascade (probably via signal-associated kinases ERK1/2 (Liu et al., 2001) and B/Akt (Kim et al., 2008) or via the phosphorylation of stress associated kinase SAPK/JNK (Xu et al., 2001)),

(iv) it can enter the cell nucleus and interact with ribonucleotides (nuclear translocation) (Xu et al., 2001).

Because of these numerous sites and modes of action, the physiology of angiogenin is quite unclear.

NO is produced by nitric oxide synthase (NOS, in our case the endothelial NOS, eNOS) from L-arginine (Palmer et al. 1988). NO is a biological transmitter mediating in particular vasodilation and angiogenesis (Fukumura et al., 1998). NO is therefore implied in a wide range of heart and vascular diseases. It has been demonstrated that nitric oxide (NO) plays a central role in angiogenesis (Fukumura et al., 1998) by triggering the proliferation of angiogenic growth factors and the differentiation of endothelial cells into vascular tube cells (Babaei et al., 1998). Ziche et al. have also shown that NO can lead to DNA synthesis and migration of coronary endothelial cells as many other angiogenic factors (Ziche et al., 1993 and 1994). In addition, NO may also promote angiogenesis through its endothelial antiapoptotic properties (Chavakis et al., 2002): angiogenesis is mainly based on proliferation of endothelial cells and therefore on inhibition of their apoptosis.

As a consequence, NO is a key factor in angiogenesis. The purpose of this study is to investigate relationships between angiogenin and extracellular NO release. Measurement of NO was performed electrochemically, using a multiple microelectrode array (MMA) developed at Imperial College London (UK) (Patel et al., 2008). Electrochemical sensors are attractive devices for biomeasurements because of robustness, linear response to concentration, ease of production and miniaturization. We have already demonstrated the utility of the MMA for NO sensing in fibroblast cells (Patel et al., 2008). To improve biocompatibility, the surface of the MMA was coated with fibronectin, an extra cellular matrix protein. Fibronectin is known to promote adequate cell adhesion and growth, and we have shown that it does not interfere with electrochemical measurements (Trouillon et al., 2009).

Firstly, (i) human umbilical vascular endothelila cells (HUVEC) and (ii) embryonic stem cells (ESC) derived endothelial cells were grown directly on the sensor ($2x10^4$ $cells.cm^{-1}$ to promote angiogenesis (Hu et al., 1997)), angiogenin was added and NO levels were monitored. Then, several inhibitors were used to study more deeply the effect of angiogenin on NO: L-NAME, RNase inhibitor and neomycin. L-NAME, an analogue of L-arginine, the eNOS substrate, is a competitive eNOS inhibitor, and blocks NO production (Rees et al., 1990). RNase inhibitor (RI) and neomycin inhibit selectively different reaction pathways of angiogenin. RI binds to the angiogenin RNase site with very high affinity ($K_i \sim 10^{-13} – 10^{-16}$ M) (Chen et al., 1997). In addition, neomycin is an antibiotic used in cancer treatment. It is known to block angiogenin nuclear translocation (Hu, 1998).

Examining the release of NO in the presence of these inhibitors should therefore elucidate the relationships between NO and angiogenin and identify the mode and site of action.

Materials and methods

Materials

All the chemicals used in this study were purchased from Sigma, unless stated otherwise, and were used without further purification. Deionized water purified through a Millipore system was used throughout all the experiments. Angiogenin was purified as previously described (Jang et al., 2004). RI was purchased from Intron Biotechnology.

Characterization of sensors

The MMA was calibrated using aliquots of authentic solution of NO. This solution was

prepared according to Feelisch and has already been described elsewhere (Patel et al., 2008). Background current was measured in degassed phosphate buffered saline (PBS, pH = 7.4) by differential pulse voltammetry (DPV), versus the MMA pseudo-gold reference. NO was then added to reach a final concentration of 75 µM. DPV was performed again to identify the peak potential.

Cell culture

HUVEC were purchased from Clonetics (San Diego, USA) and cultivated in EBM-2 medium (Clonetics) on gelatin coated 75 ml flasks, at 37°C, 95% O_2, 5% CO_2. Cells from passage 6 to 9 were used.

ESC-derived endothelial cells were prepared by mechanical isolation and cell sorting (Cho et al., 2007).

Modification and preparation of the sensors

The MMA were modified using a sylgard (Dow Corning) custom-made reaction cell to allow deposition of a 500 µl volume on the sensor. A lid made from a Petri dish was also fitted on the cell to minimise evaporation.

Prior to any experiment, biological debris was removed using trypsin solution. Trypsin was deposited into the cell and incubated it at 37°C for one hour. They were then rinsed with 70% ethanol followed by water. The gold electrodes were electrochemically cleaned by performing cyclic voltammograms between 1 V and -0.8 V at 0.5 $V.s^{-1}$ in 0.1 M sulphuric acid until stability of the graphs. The electrodes potential was then held at –0.6 V to reduce gold oxides.

The sensor was then sterilized with 70% ethanol and UV light and rinsed with PBS (pH = 7.4). 100 µl of human fibronectin (20 $\mu g.ml^{-1}$, in DMEM) was deposited on the sensor and let to dry. The excess of fibronectin was rinsed with PBS.

Electrochemical measurements

Cells were harvested using trypsin and counted with a cytometer and Trypan blue. 1000 cells were suspended in 500 µl of EBM-2 medium and deposited on the sensor. The chip was then incubated for at least 2 hours.

A DPV was then recorded between 0 V and 1.5 V vs Ag/AgCl.

2 µl of bovine angiogenin (5.26 $mg.ml^{-1}$ in deionized water) were added with the relevant inhibitor if required (protocol described below) to reach a final concentration of 21 $\mu g.ml^{-1}$. The MMA was placed in the incubator for one hour and another DPV was recorded.

Inhibitors

For the L-NAME study, the cells were maintained in culture medium containing 100 µM L-NAME during at least one hour before any measurement.

Similarly, in the neomycin setup, cells were cultivated for at least one hour in medium containing 50 µM neomycin before any measurement.

The RNase inhibitor was mixed with angiogenin (1:2 mass ratio) and incubated at 37°C for one hour. This mixture was then added to the cell medium to reach a final angiogenin concentration of 21 $\mu g.ml^{-1}$ and incubated for 1 hour.

Data processing

The DPV results were processed by normalizing the peak current obtained after angiogenin injection by the peak current measured before injection. This normalization guaranteed consistency between the different measurements by minimizing the effect of variations in size, background species, heterogeneity in cell population. The results obtained for each experimental condition were then averaged, and the means compared using Student's t-tests.

Results

Measurement of nitric oxide and other nitrous compounds

Fig.1 shows curves obtained for various dissolved NO solutions. We notice a sharp peak at 0.6V. This peak is due to NO oxidation and increases with NO concentration.

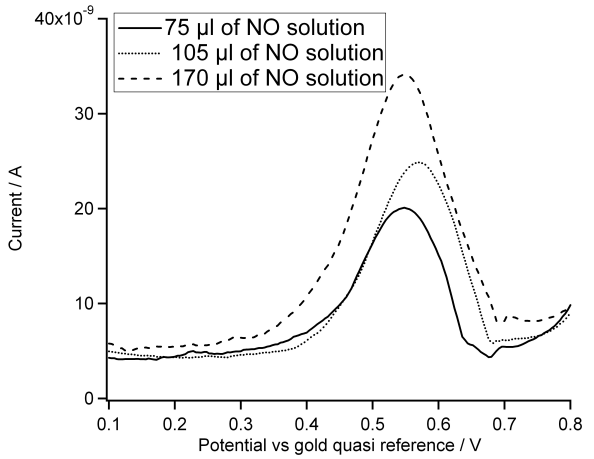

Figure 1: Differential pulse voltammograms in PBS, pH = 7.4, with increasing concentration of nitric oxide

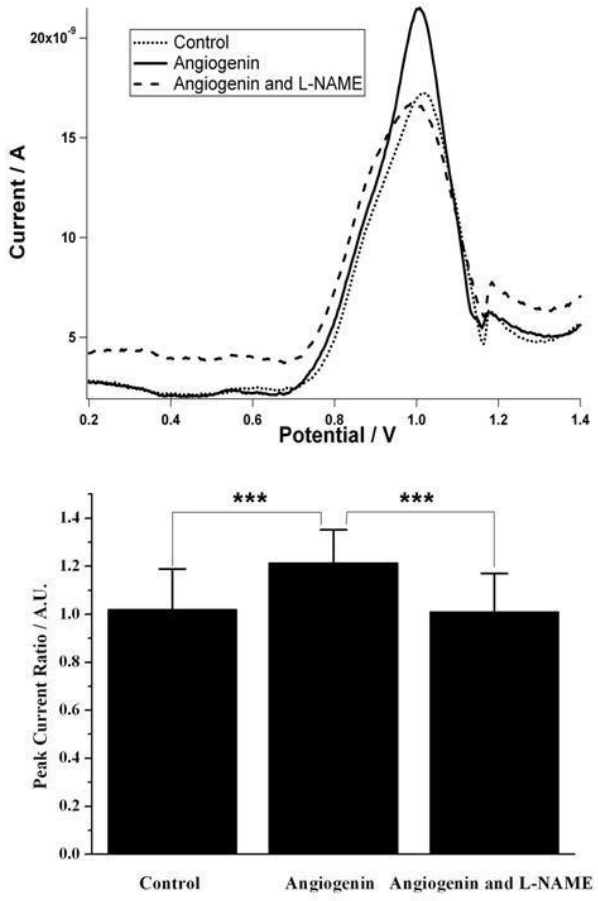

Figure 2: Differential pulse voltammograms in EBM-2 medium in presence of HUVEC, with angiogenin, in presence ( ) or absence of L-NAME (—), and control without angiogenin and L-NAME (·····) (top); Results for the differential pulse voltammograms performed in EBM-2 medium in presence of HUVEC, with angiogenin, in presence or absence of L-NAME, and control without angiogenin and L-NAME ($n \geq 17$, ***: $p < 0.001$) (bottom)

Effect of angiogenin

Fig.2 displays typical voltammograms obtained from HUVEC measurements.

Results for control setup and after angiogenin addition, in presence or absence of L-NAME, are also presented. We see similar peaks to the ones obtained for pure NO solutions. In particular, we see that if L-NAME is present, the response is very similar to the one measured in the control experiment. However, if only angiogenin is added to the medium, we notice an increase in peak magnitude. These results are summarized on Fig.2, which presents the ratio of the peak magnitude after and before addition of angiogenin. There is a significant increase in peak height if only angiogenin is present (+ 21%, $p < 0.0001$, $n \geq 17$). However, if the HUVEC were previously incubated 100 µM L-NAME, we do not see any significant change in peak magnitude ($p > 0.8$, $n \geq 17$).

As a control, the same protocol was applied to stem cell derived endothelial cells (Fig.3). We obtained exactly similar results, angiogenin leading to an increase in the peak current (+22 %, $p<0.0001$, $n \geq 18$), and L-NAME inhibiting this increase.

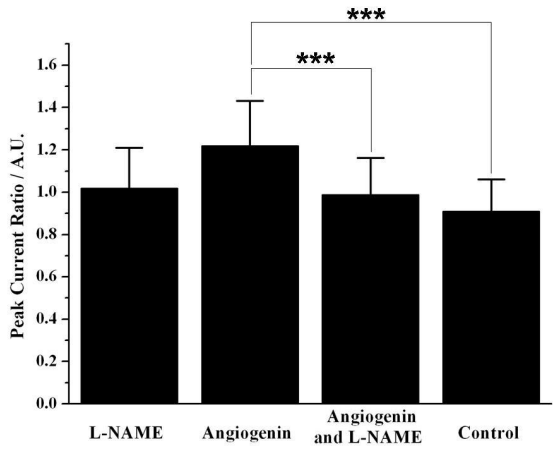

Figure 3: : Results for the differential pulse voltammograms performed in EBM-2 medium in presence of ESC derived endothelial cells, with angiogenin, in presence or absence of L-NAME, and controls without angiogenin and L-NAME, and with L-NAME only ($n \geq 18$, ***: $p < 0.001$)

Effect of inhibitors

To understand the physiological pathway of angiogenin, several selective inhibitors have been used in the same kind of experiment

a) RNase inhibitor

Fig.4 shows the statistical results obtained for addition of angiogenin in presence or absence of RNase inhibitor (RI). Angiogenin alone led to an increase in magnitude of 22% ($p<0.001$, $n \geq 10$) and addition of a mixture of RI and angiogenin increased the peak magnitude by 17% ($p<0.001$, $n \geq 10$). The increases observed for both treatments are not statistically significantly different ($p > 0.45$, $n \geq 10$).

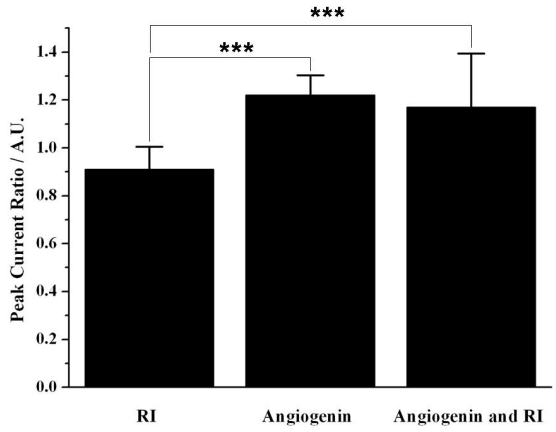

Figure 4: Results for the differential pulse voltammograms performed in EBM-2 medium in presence of HUVEC, with angiogenin, in presence or absence of RI, and control without angiogenin and with RI ($n \geq 10$, ***: $p < 0.001$)

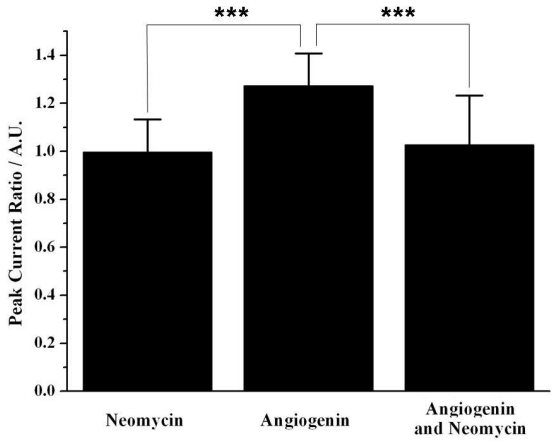

Figure 5: Results for the differential pulse voltammograms performed in EBM-2 medium in presence of HUVEC, with angiogenin, in presence or absence of neomycin, and control without angiogenin and with neomycin ($n \geq 10$, ***: $p < 0.001$)

b) Neomycin

Fig.5 shows the results obtained for this experiment. Again, if we add angiogenin to the cell medium, we see an increase in current peak (+ 27%, $p < 0.001$, $n \geq 10$). However, if the cells were previously incubated with neomycin, no significant increase is measured ($p < 0.55$, $n \geq 10$). Neomycin inhibits the angiogenin induced increase in peak current

Discussion

Effect of angiogenin on nitric oxide synthase activity

We have shown that adding angiogenin to cell medium led to a higher current peak. In addition, this peak is very similar to the one obtained previously in pure NO solutions and is expected to be due to an increase in NO concentration. This NO is expected to be produced by the HUVEC, NOS activity being probably triggered by angiogenin. This assertion was confirmed by the L-NAME tests. As an analogue of L-arginine, L-NAME is known to inhibit competitively NOS and therefore NO production. By adding L-NAME to our setup, NOS in our population of HUVEC is inhibited and no current increase is observed. This shows that this increase in peak current was due to NOS activity and that the peaks we measure are due to NO oxidation. Furthermore, these experiments show that angiogenin triggers NOS activity and leads to increases in extracellular NO.

The same results were obtained when the same experiment was carried out in stem cell derived endothelial cells as a control showing that angiogenin induced NOS activation is a general feature of endothelial cells.

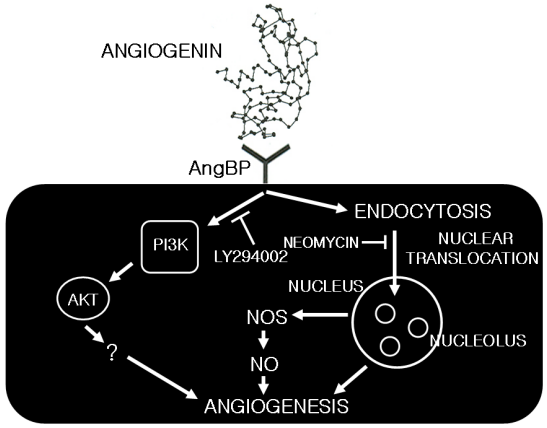

Figure 4: A hypothetical scheme for the mechanism of action of angiogenin modified from Kim et al. (2008)

Physiological pathway of angiogenin

The physiological pathway leading to NOS activation was studied using 2 angiogenin inhibitors: RI and neomycin. We do not see any effect of RI, indicating that angiogenin induced eNOS activity does not depend on the RNase activity. However, neomycin completely inhibits the NO increase. Neomycin blocking nuclear translocation, this result shows that eNOS is activated through angiogenin interactions with cell nucleus. A modification of hypothetical scheme for the mechanism of action of angiogenin is shown in Fig. 6. This conclusion has to be related to the fact that nuclear translocation of angiogenin is a general requirement for angiogenesis (Kishimoto et al., 2005).

Conclusion

We have demonstrated that angiogenin leads to enhanced release of NO. Interaction of angiogenin with the cell nucleus is a critical requirement for this angiogenin induced eNOS activity, which tends to prove that nuclear translocation of angiogenin is a major event in angiogenesis. Selective inhibitors demonstrate that nuclear translocation is required for angiogenin mediated NO release.

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