Cardiac endothelium: More than just a barrier

Hans Strijdom and Amanda Lochner

Dept Biomedical Sciences (Division Medical Physiology),
Faculty of Health Sciences, University of Stellenbosch

Address for correspondence:
Dr Hans Strijdom
Dept of Biomedical Sciences
Division of Medical Physiology
Faculty of Health Sciences
University of Stellenbosch
Tygerberg
7505
South Africa

Email:
jgstr@sun.ac.za

ABSTRACT

Cardiac endothelium consists of highly specialised endothelial cells that are custom-designed to influence and regulate myocardial function. Various bioactive molecules, such as nitric oxide (NO) are released and given the short diffusion distance between especially the myocardial capillary endothelial cells (cardiac micro vascular endothelial cells, CMECs) and adjacent cardiomyocytes, an ideal microenvironment is created for paracrine communication. In this review paper, the relative role of the CMECs with regard to NO generation, the mechanisms of NO generation, and possible consequences of the released NO are described. Particular attention is given to these parameters under conditions of oxygen deficiency, as this is one of the most common pathophysiological conditions affecting the heart. SAHeart 2009; 6:174-185

INTRODUCTION

Traditionally, the endothelium was viewed as a selectively permeable barrier between the circulating blood and underlying subvascular interstitium; however, it is now known that the endothelium has a far more intricate role: it is in fact a metabolically active organ, with endothelial cells acting as receptor-effector homeostats in the vasculature.[1,2] In this respect, endothelial cells secrete a variety of vaso-active and other bioactive molecules that regulate biological processes such as vascular tone, vascular inflammation and haemostatis[1,3] (Figure 1).

Although the role of endothelial cells in the maintenance of homeostasis in the general vascular system is well described, studies investigating their role in the myocardium, where they are numerically very well represented and often within diffusion distance from the cardiomyocytes, are less abundant. It is the proximity between cardiac endothelial cells and cardiomyocytes that is of particular importance, since it allows for paracrine communication between these cell types.[4,5] Therefore, cardiac endothelial cells are strategically positioned and functionally geared towards being major regulators of myocardial function,[4] in both the physiological setting and the pathophysiological setting such as occurs with deficient oxygen supply (ischaemia and hypoxia). Despite our awareness of the presence of these metabolically active cells in the heart, it is surprising to note that many studies continue to define cardiac function in terms of[4]

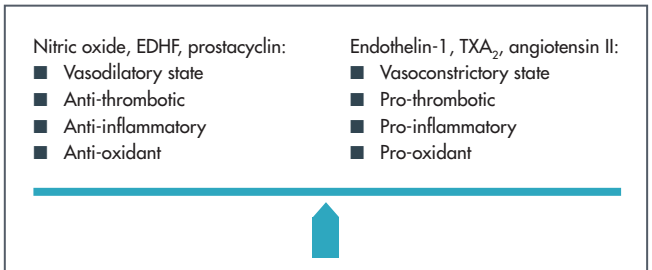


FIGURE 1: The role of endothelium in vascular homeostasis. Various factors released by vascular endothelial cells result in equilibrium between a vasodilatory state (including anti-thrombotic, anti-inflammatory and anti-oxidant effects) and a vasoconstrictory state (including pro-thrombotic, pro-inflammatory and pro-oxidant effects). Vasodilatory effects are induced by factors such as nitric oxide (NO), endothelium-derived hyperpolarising factor (EDHF) and prostacyclin, whereas vasoconstrictory effects are induced by endothelin-1, thromboxane A2 (TXA_2) and angiotensin II. When homeostasis is lost in favour of a net vaso-constrictory state, endothelial dysfunction will ensue.

cardiomyocyte physiology and function, coronary perfusion and neuroendocrine regulation.[5]

Nitric oxide (NO) is one of the most important signalling molecules produced and released by endothelial cells throughout the body via the constitutively expressed enzyme, endothelial NO synthase (eNOS).[6] Apart from its well known vascular effects, including vasodilation, anti-thrombosis and anti-inflammation,[7,8,9] NO is known to protect the myocardium against ischaemic/hypoxic injury.[10,11] The two best described NO-releasing cell types in the heart are the cardiac endothelial cells and cardiomyocytes and since the former outnumber the latter by 3:1,[4,5] it is fair to assume that cardiac endothelial cells are the predominant source of cardiac NO and by implication, a putative source of NO-derived protection in the heart. It is therefore important and relevant that more studies investigate the role of cardiac endothelial cells as a source of NO in the normal and hypoxic/ischaemic myocardium. Despite a plethora of studies on cardiac NO, data on the relative importance of NO derived from cardiac endothelial cells and cardiomyocytes are surprisingly scant.[12]

ENDOTHELIAL SUBTYPES IN THE HEART

Endothelial cells in the heart can be classified based on their effects on, and proximity to, cardiomyocytes. Therefore, the endothelial cells that line the myocardial capillaries (cardiac microvascular endothelial cells, CMECs) and the endocardium (endocardial endothelial cells, EECs) are collectively referred to as cardiac endothelium, as they are in close proximity to adjacent cardiomyocytes, and consequently have direct effects on cardiomyocyte function.[5] Conversely, the endothelial cells that line the larger coronary arteries and veins are located further away from the cardiomyocytes. The coronary vascular endothelial cells, as they are called, exert indirect effects on myocardial function by controlling coronary perfusion.[5] The classification of endothelial cells in the heart is shown in Figure 2. The EECs occupy a large cavity surface area to chamber volume ratio, which effectively allows for exposure to 100% of the circulating blood.[5,13] Given their exposure to the total amount of circulating humoral factors, the EECs are likely to act as a sensing system, in addition to their role as paracrine regulators of cardiomyocyte function.[13] Interestingly, in a study on quail embryos, it was found that EECs and cardiomyocytes initially develop from the same cardiac mesodermal region, although they eventually separate from each other.[14] In fact, in early embryonic development, the EECs are the only endothelial cell subtype present in the primitive spongious heart tube, where they exist alongside the cardiomyocytes, in which, at around this time, the very first contractions begin to appear.[13] The discovery that embryonic EECs release neuregulin, a member of the epidermal growth factor family, supported speculation of an obligatory role for EECs in cardiomyocyte growth and development, especially since it was also[6] shown that the embryonic cardiomyocytes express the neuregulin receptors, ErbB2 and ErbB4.[15]

In addition to its role as a sensing system of circulating blood and its contents and paracrine regulator of myocardial development and function, the endocardial endothelium is thought to represent a blood-heart barrier (BHB), similar to the well described blood-brain endothelial barrier.[5] The subendocardial region contains highly excitable tissues including the Purkinje fibre network and cardiomyocytes, which requires fine control of ionic concentra-

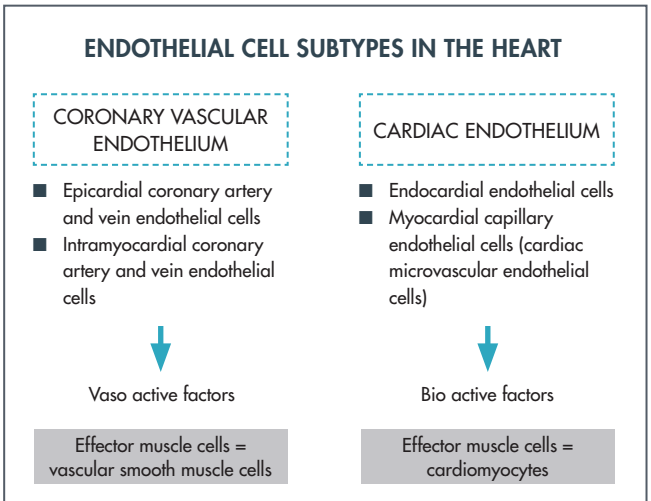


FIGURE 2: Classification of endothelial cells in the heart. The classification is based on the proximity to, and direct paracrine influence on cardiomyocytes. Coronary vascular endothelial cells are situated in the larger coronary arteries and veins, and have no direct effects on cardiomyocyte function. Their biological effects are similar to those of endothelium elsewhere in the body (maintenance of vascular homeostasis). Conversely, the cardiac endothelial cells (endocardial endothelial cells and cardiac micro-vascular endothelial cells) have greater direct effects on cardiomyocyte function via the release of paracrine messengers such as NO.

tions and fluxes via the BHB in order to maintain cardiac rhythmicity and mechanical performance.[5] Endocardial endothelial cells are well suited for this function through the presence of tight and gap junctions and through their distinct electrophysiological properties (high concentration of membrane ion channels and Na+ - K+ -ATPase).[5] EECs demonstrate a high concentration of eNOS expression in the Golgi bodies; furthermore, staining studies have shown that the Golgi bodies in EECs are significantly larger in size than in other endothelial cell types.[13] These properties point to a higher metabolic capacity in EECs compared to vascular endothelial cells.[5] Compared to the EECs, the appearance of the second cardiac endothelial cell subtype, the CMECs, is a relatively late event in the embryonic development of the heart, and their ability to influence cardiomyocyte function appears even later.[13] In this review paper, we will focus on the CMECs, their relation to the underlying cardiomyocytes and their role as an important source of NO in the heart.

THE UNIQUE STRUCTURAL AND FUNCTIONAL RELATIONS BETWEEN CARDIAC MICROVASCULAR ENDOTHELIAL CELLS AND CARDIOMYOCYTES

Despite occupying the majority of the heart's volume (~ 75% of total tissue volume), and having a cell-to-cell mass ratio of 25:1 compared to cardiac endothelial cells, the cardiomyocytes represent numerically less than 40% of the total cell number in the heart.[4,5] In fact, it is proposed that there are 3 cardiac endothelial cells for every cardiomyocyte.[4] Anatomically, the architecture of the heart promotes close proximity between the cardiomyocytes and the cardiac endothelial cells, particularly the CMECs. The myocardial capillary network is vast with capillaries located strategically around the cardiomyocytes allowing for each cardiomyocyte to be surrounded by 3-4 capillaries.[4] Despite the relatively large size of adult mammalian cardiomyocytes (~10–100 µm), the average intercapillary distance is ~10–50 µm, which results in an intricate endothelial cell-cardiomyocyte assembly.[4,5,16] In view of this unique structural arrangement, each CMEC is within 1 µm distance from an underlying cardiomyocyte, which creates an ideal micro-environment for local transport and molecular communication.[5] The CMEC-cardiomyocyte arrangement in the myocardium is depicted in Figure 3.[8]

Unlike the EECs, CMECs only receive ~ 3-5% of the circulation, which, by implication, suggests that the relative concentration and pressure gradient of the humoral factors, rather than their total amount, would be more important for optimal paracrine communication (e.g. diffusion).[13] CMECs, as endothelial cells elsewhere in the body, release various bioactive molecules, the most important being NO, endothelin-1 (ET-1) and prostacyclin (PGI2).[4,5,13] Collectively, these CMEC-derived factors have direct effects on cardiomyocyte function, including cardiomyocyte contraction, rhythmicity and growth.[5] The molecular communication between CMECs and cardiomyocytes is bi-directional, which implies that the regulatory actions between these cell types are reciprocal. The paracrine communication between CMECs and cardiomyocytes is depicted in Figure 4.

CMEC-derived ET-1 acts in an autocrine and paracrine manner.[4] Autocrine effects are achieved when ET-1 binds to ETB receptors expressed on CMECs leading to NO and prostacyclin release.[4] Conversely, paracrine effects are observed when ET-1 binds to ETA receptors on cardiomyocytes resulting in myocardial contraction.[4] When released from vascular endothelial cells, prostacyclin (PGI2) has anti-clotting and vasodilatory actions. However, in the myocardium, CMEC-derived PGI2 (and the more abundant EEC-derived PGI2) acts in a reciprocal fashion with NO: when endogenous PGI2 release is stimulated, the inotropic effects of

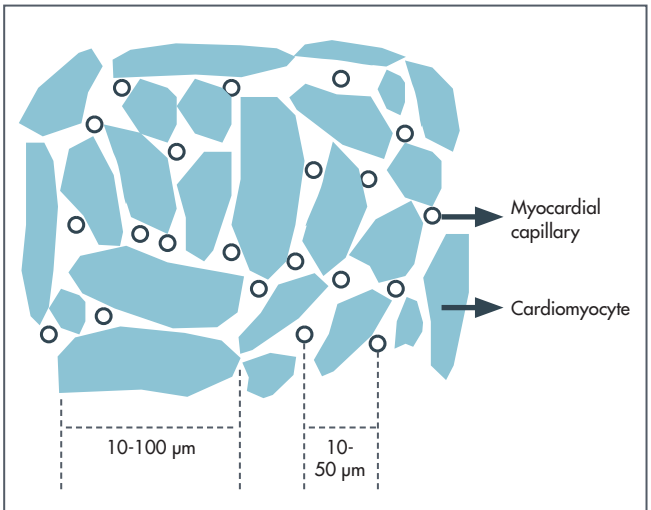


FIGURE 3: The CMEC-cardiomyocyte arrangement in the myocardium. Cardiomyocytes (~10-100 µm in size) are surrounded by myocardial capillaries (average intercapillary distance ~10-50 µm). Each cardiomyocyte is associated with at least 3-4 capillaries.

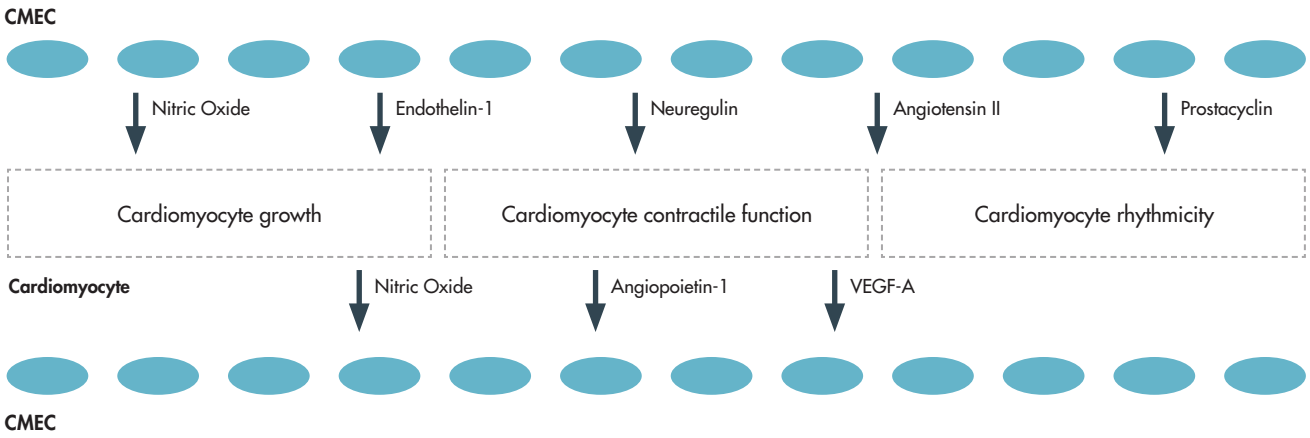


FIGURE 4: Paracrine communication between CMECs and cardiomyocytes. CMECs have direct paracrine effects on cardiomyocyte growth, contractile function and rhythmicity via the release of several bioactive molecules such as NO, endothelin-1, angiotensin II, prostacyclin and neuregulin. Cardiomyocytes also release factors that affect CMEC function, including NO, angiopoietin-1 and vascular endothelial growth factor A (VEGF-A).

NO are abolished while NO synthesis inhibition results in PGI2-induced positive inotropic effects. [5]

CMECs AND CARDIOMYOCYTES AS CELLULAR SOURCES OF MYOCARDIAL NO PRODUCTION

Both CMECs and cardiomyocytes are able to synthesise and release nitric oxide (NO), one of the most important chemical messengers in the heart;[5] NO is a key regulator of excitation-contraction coupling in the cardiomyocytes and therefore of myocardial contractile function.[17] NO has also been shown to modulate heart rate, ß-adrenergic inotropic response, myocardial energetics and substrate metabolism.[12] NO effects are achieved via intracellular downstream mechanisms that have initially been thought to be mainly cyclic GMP (cGMP)-mediated, although it is now known that many of the effects of NO are cGMP-independent.[17] The contractile effects of NO depend on the intracellular location of its release and the end-targets of its signalling pathways, e.g. when NO signalling targets the L-type calcium channels in cardiomyocytes, a decreased calcium current ensues which leads to attenuated ß-adrenergic receptor stimulated contraction.[17] In addition to its effects on myocardial contractile function and metabolism, NO is regarded as a potent cardioprotective molecule, particularly in the context of ischaemia-reperfusion injury and delayed ischaemic preconditioning.[10,11] In view of the major functional and protective effects that NO exerts in the heart, as well as its unique properties as an efficient local messenger molecule (NO is a gas and free radical, and therefore highly diffusible and reactive),[18,19] it is imperative[10] that more studies focus on the cellular sources of cardiac NO with particular emphasis on those cell types between which paracrine communication is likely. The enzymatic generation of NO in CMECs and cardiomyocytes is derived from the three most extensively described NO synthase (NOS) isoforms to date, viz. endothelial NOS (eNOS), inducible NOS (iNOS) and neuronal NOS (nNOS), all of which are to a larger or lesser extent expressed in both cell types.[20] Most authors agree that the constitutively expressed eNOS is associated with continuous NO production in relatively low quantities (< 100 nM) under physiological, baseline conditions.[7,21] However, very few studies have quantified and compared the relative expression of eNOS in CMECs and cardiomyocytes, although immune-histochemical studies have suggested higher expression in CMECs.[5,12] In a study using immune-fluorescence and confocal microscopy, a significant degree of non-uniformity was found with regard to the distribution of eNOS expression within the different cell subtypes of the cardiac endothelium: EECs showed greater eNOS staining compared to CMECs.[22]

iNOS (induction by factors such as inflammatory cytokines), is expressed in both cardiac endothelial cells and cardiomyocytes, although the latter demonstrate a relatively higher iNOS / eNOS ratio than endothelial cells.[21]

iNOS releases NO in quantities much greater than eNOS (>1 µm).[21] NO derived from iNOS is often associated with pathophysiological conditions; however the harmful effects are not due to NO per se, but rather the ability of excessive amounts of NO released by iNOS to react with superoxide to form the highly reactive peroxynitrite radical.[23] From a clinical point of view, excessive NO formation is an important mechanism in the development of multiple organ failure (including myocardial depression) associated with sepsis.[24] NOS inhibition studies in large animal models have revealed inconclusive data as to whether any one specific NOS isoform is involved, rather suggesting that both iNOS and nNOS derived NO may be involved in the pathophysiological processes of septic shock.[24] Despite the uncertainty, administration of non-specific NOS inhibitors have shown promising therapeutic effects by reversing sepsis-induced hemodynamic complications.[24]

In contrast, iNOS-derived NO may also be cardio-protective as it has convincingly been shown to be a mediator of protection in ischaemic preconditioning.[10,11] There is not much data available on the role of nNOS in endothelial cells, although more and more evidence is appearing that demonstrates a significant expression of, and role for nNOS in cardiomyocytes.[17]

ENOS IN CMECs: MANY QUESTIONS REMAIN

It is widely accepted that eNOS is the predominant enzymatic source of NO in endothelial cells, including the CMECs. As explained earlier, CMECs do exhibit positive staining for eNOS, albeit to a lesser degree compared to other forms of cardiac endothelial cells such as the EECs.[22] Given the potential importance of reciprocal cellular cross-talk between CMECs and the underlying cardiomyocytes, it is surprising that the majority of studies in this field rely on semi-quantitative staining techniques to assess the relative expression of eNOS in these two cell types. It is also apparent from the literature that very few studies make use of direct intracellular NO-measurement tools to quantify, or at least compare, the amount of NO produced by each cell

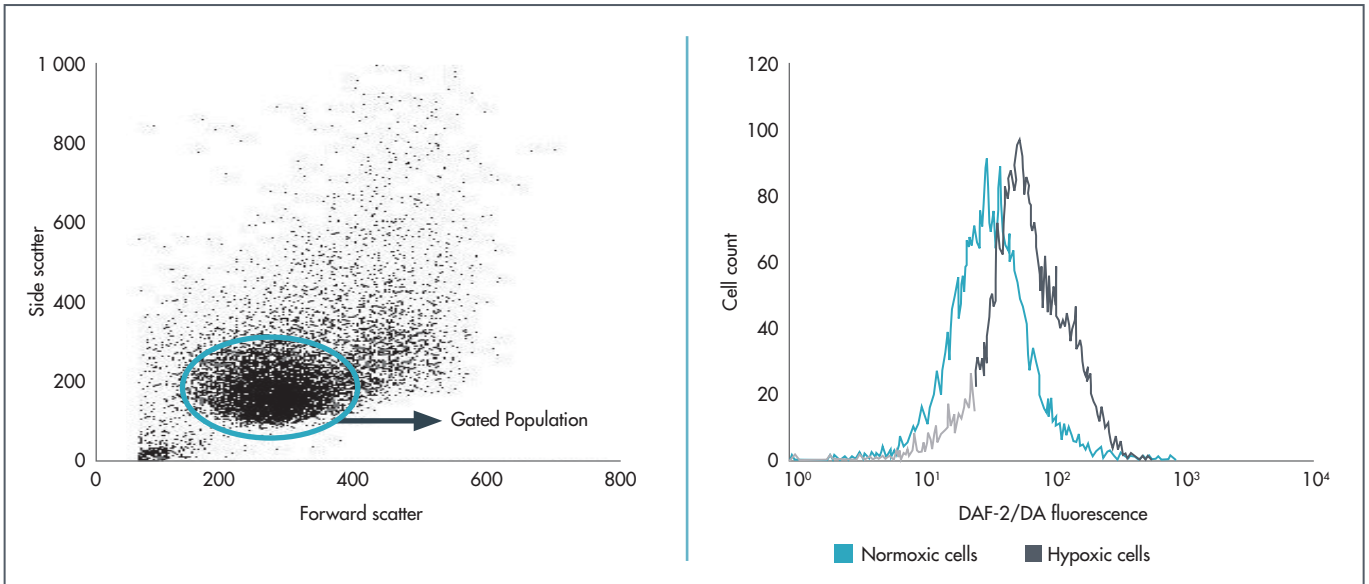


FIGURE 5: Direct intracellular NO measurement by flow cytometric analysis of DAF-2/DA fluorescence. CMECs are pre-treated with the NO-specific probe, DAF-2/DA (100 µm) which remains present for the duration of the experiments, after which the probe is washed out and samples analysed by flow cytometry (Becton Dickinson FACSCalibur® Analyzer with the aid of Cellquest® Version 3.3 software.)

A: The cell population of interest is gated based on the forward scatter (cell size) and side scatter (cell granularity) properties of the CMECs, indicated by the blue circle on the scatter plot.

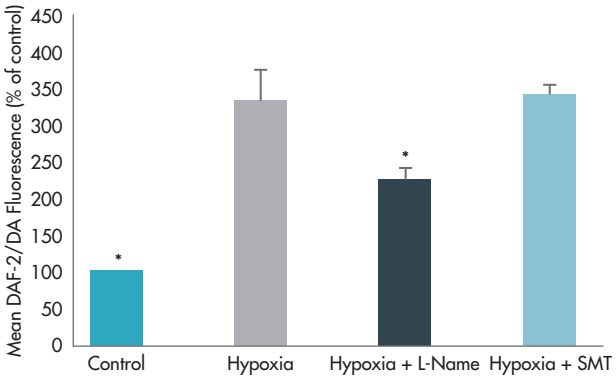
B: The fluorescence intensity (emitted when DAF-2/DA reacts with NO) of the gated population is measured in the FL-1H (green fluorescence) channel. From the histogram it is clear that there is an increase in fluorescence intensity in hypoxic CMECs compared to normoxic CMECs, indicating an increase in intracellular NO production.

type.[25] Without direct, quantitative measurements, it will always be difficult to reach any substantial conclusions regarding the existence or importance of paracrine NO communication between CMECs and cardiomyocytes both with regard to their ability to generate NO (i.e. eNOS measurements) and the amount of NO they produce (i.e. direct NO-specific measurements).[26] In addition to the hiatus that exists regarding the relative expression of eNOS protein, few studies have investigated the regulation of eNOS activity (via phosphorylation) in CMECs; in fact, the role of eNOS activation by phosphorylation is under-researched in heart tissue as a whole.[27] One of the most common pathophysiological conditions affecting the myocardium is the development of reduced oxygen supply (ischaemia and hypoxia). It is now widely accepted that myocardial NO content increases shortly after the onset of ischaemia/hypoxia, and that this increase is partly due to NOS activation; however, considerable uncertainty still remains regarding which NOS isoforms are involved.[21,28] Apart from NOS involvement in hypoxia/ischaemia-induced NO production, other NOS-independent sources are also implicated, such as the reduction of nitrites to NO.[29]

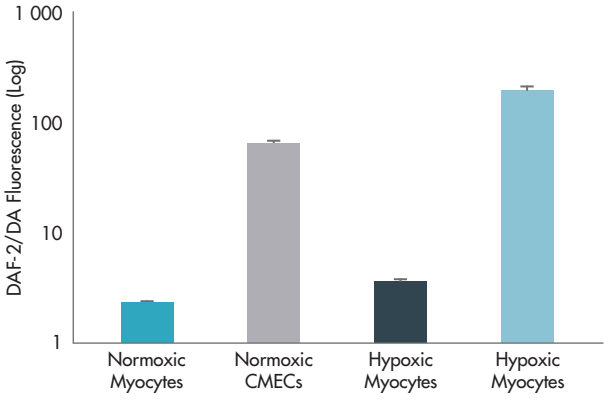
NO PRODUCTION IN CMECS

In order to gain more knowledge on the role of CMECs in the myocardium with regard to NO production, we studied intracellular NO generation in a model of cultured CMECs derived from adult rat hearts (and compared findings with those obtained in isolated cardiomyocytes). NO production was measured in normoxic and hypoxic CMECs using a fluorescence-based detection technique previously developed in our laboratory (DAF-2/DA: NO-specific fluorescent probe)[25,26] (Figure 5).

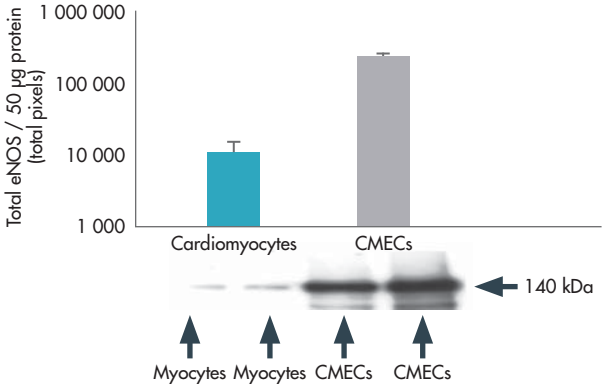
Most authors agree that myocardial NO levels increase during hypoxia [Schulz 2004] and our model of cultured CMECs also responded in this fashion by demonstrating up to 3.3-fold increase in intracellular NO production[26] (Figure 6A). This is in agreement with other studies that investigated hypoxia-induced NO production in endothelial cells obtained from the heart, viz. coronary artery, arteriolar and micro-vascular endothelial cells.[30,31,32] Interestingly, not all endothelial cell studies observe hypoxia-induced increases in NO production: one study on human saphenous vein endothelial cells showed a 74% reduction in NO levels after exposure



A: Bar chart depicting NO production under normoxic and hypoxic conditions, with and without the administration of NOS inhibitors, NW-nitro-L-arginine methyl ester (L-Name, 50 µM; non-specific NOS inhibitor) and S-methylisothiourea (SMT, 100 µm; iNOS-specific inhibitor). CMECs subjected to hypoxia (hypoxic pelleting) showed increased NO production, L-Name significantly reduced NO production and SMT had no effect. *: *p < 0.05 vs. hypoxia.*



B: Bar graph showing NO production in CMECs and cardiomyocytes comparing data on a cell-to-cell basis. Under normoxic conditions, CMECs produced 26 times more NO than an equal number of cardiomyocytes and in hypoxia; CMECs produced 52 times more NO than cardiomyocytes.



C: Expression of total eNOS protein (measured by Western blotting analysis; molecular weight 140 kDa) in normoxic CMECs and cardiomyocytes. CMECs expressed 22 times more eNOS protein than cardiomyocytes.

FIGURE 6: NO production and eNOS protein expression in CMECs.

(Blots and graphs reproduced with the permission of FASEB Journal and obtained from: Strijdom H, Jacobs S, Hattingh S et al. Nitric oxide production is higher in cardiac micro-vessel endothelial cells than ventricular cardiomyocytes in baseline and hypoxic conditions: a comparative study. FASEB J 2006;20(2): 314-316.)

to hypoxia.[33] These discrepancies may be explained by differences in the endothelial cell subtype investigated and the protocols used.

Of particular interest to us was to confirm previously held assertions that CMECs generate more NO than cardiomyocytes. In order to achieve this objective, it was necessary to assess NO production with a technique that allowed for direct measurement of intracellular NO levels, since opinions at the time were mainly based on data obtained from indirect immunohistochemical and fluorescence based labelling studies directed at eNOS protein.[5,12] The flow cytometry-based NO-detection technique developed in our laboratory made it possible to gate populations of identical cell numbers (See Figure 5 for explanation of flow cytometry technique), which enabled the investigator to calculate and compare data on a cell-to-cell basis. Our findings validated the prevailing belief that CMECs generate more NO than cardiomyocytes: the CMECs produced 26 times more NO/cell than cardiomyocytes under normoxic conditions (Figure 6B). Further validation for the NO data was found when we compared normoxic eNOS protein expression (measured by Western blotting analysis) in the two cell types: baseline eNOS expression was 22 times greater in the CMECs on a cell-to-cell basis (Figure 6C). When subjected to an identical hypoxia protocol, CMECs generated 52 times more NO/cell than cardiomyocytes[26] (Figure 6B). To our knowledge, this was the first study to directly measure and compare intracellular NO production in these two cardiac cell types. The importance of these findings lies in the fact that it creates a better understanding of the possible nature of the in vivo myocardial NO dynamics. The CMECs, of all the endothelial cell types in the heart, are in closest proximity to the largest portion of cardiac muscle cells, and given the fact that there is at least one CMEC for every cardiomyocyte, there is a high probability that the cardiomyocytes could be recipients of excess NO released by the CMECs (termed "spill-over diffusion").[26] Preliminary studies in our laboratory (data not shown) have suggested that there is a net uptake of NO by cardiomyocytes when co-cultured with CMECs, however, more research is required to confirm this phenomenon as well as to establish the biological effects of spill-over diffusion, not only under normoxic conditions, but more specifically in a hypoxic environment.

REGULATION AND ACTIVATION OF ENOS IN CMECS

Following the observation that NO generation increases after exposure to hypoxia, we wanted to determine the source of such an increase, especially since this has not yet been well established in CMECs. Our primary focus was on eNOS and iNOS as putative enzymatic sources of NO production. Initially, we inhibited eNOS and iNOS pharmacologically during hypoxia with L-NAME (relatively non-specific NOS inhibitor) and SMT (iNOS-specific inhibitor) to tease out NOS isoform involvement. Results suggested a partial contribution from eNOS, but that iNOS did not seem to be involved at all[26] (Figure 6A). In fact, subsequent Western blotting measurements confirmed the iNOS inhibition data, as it revealed that the iNOS protein was not expressed in our CMEC model at all.[28]

From the above data, it seems that CMECs (and endothelial cells from the larger blood vessels in the heart as demonstrated by other authors) respond to hypoxia by increasing their NO production, and that this is partly mediated by increased eNOS activity. On all accounts, the iNOS isoform does not seem to be involved in our model of CMECs. It is well known that data obtained from studies using pharmacological inhibitors can be notoriously misleading, since many inhibitors are non-specific. In addition, existing data on the regulation of eNOS protein expression and activation by phosphorylation in CMECs (and the whole heart for that matter) are scant,[27] particularly with regard to the role of hypoxia as a putative activating stimulus.[12,34] Phosphorylation of eNOS at the Ser 1177 residue is the best described and probably most important mechanism of eNOS activation.[35,36] We therefore, as a next step, repeated the normoxia and hypoxia investigations and measured total eNOS expression and activated eNOS (phosphorylated eNOS at Ser 1177) levels by Western blotting analyses.[28] The data showed that total eNOS protein expression increased by 4-fold in CMECs exposed to 18 h of low PO_2 incubation (see legend of Figure 7 for a description of this hypoxia protocol), and that activated levels of eNOS increased by 5-fold[28] (Figure 7A). The relative activated/total eNOS increase was therefore 1.25-fold, implying that the activated levels of eNOS increased by a greater margin than the total protein in this hypoxia protocol. In CMECs exposed to 60 min hypoxic pelleting (See legend of Figure 7 for

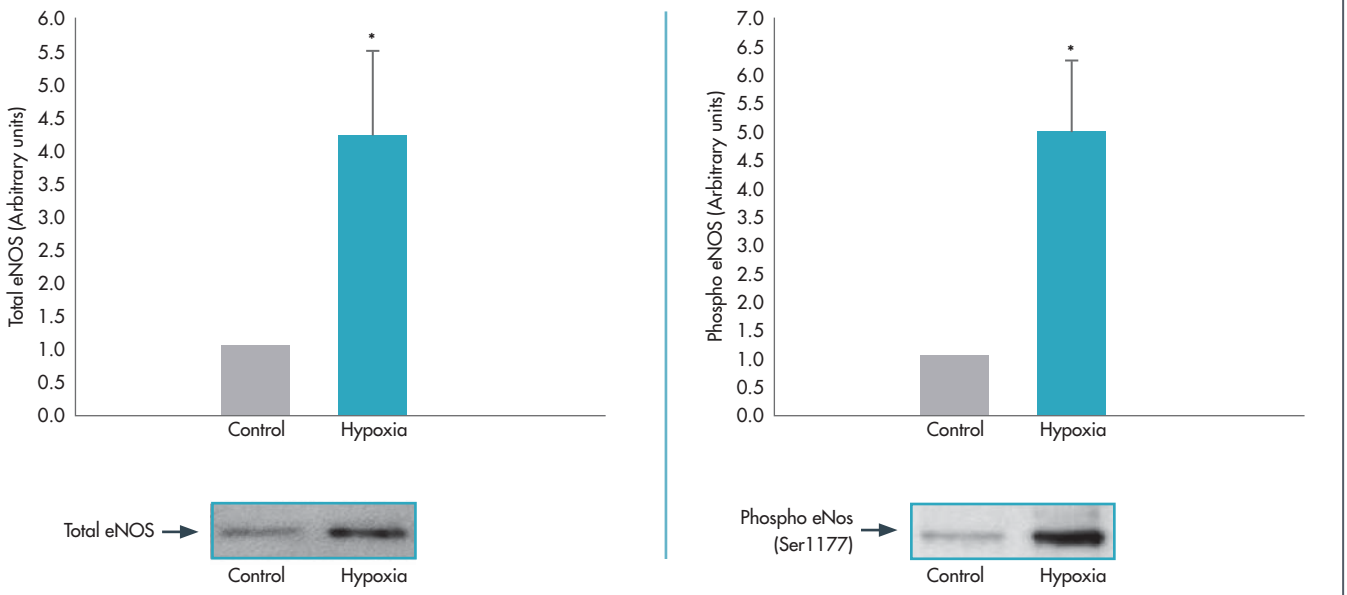
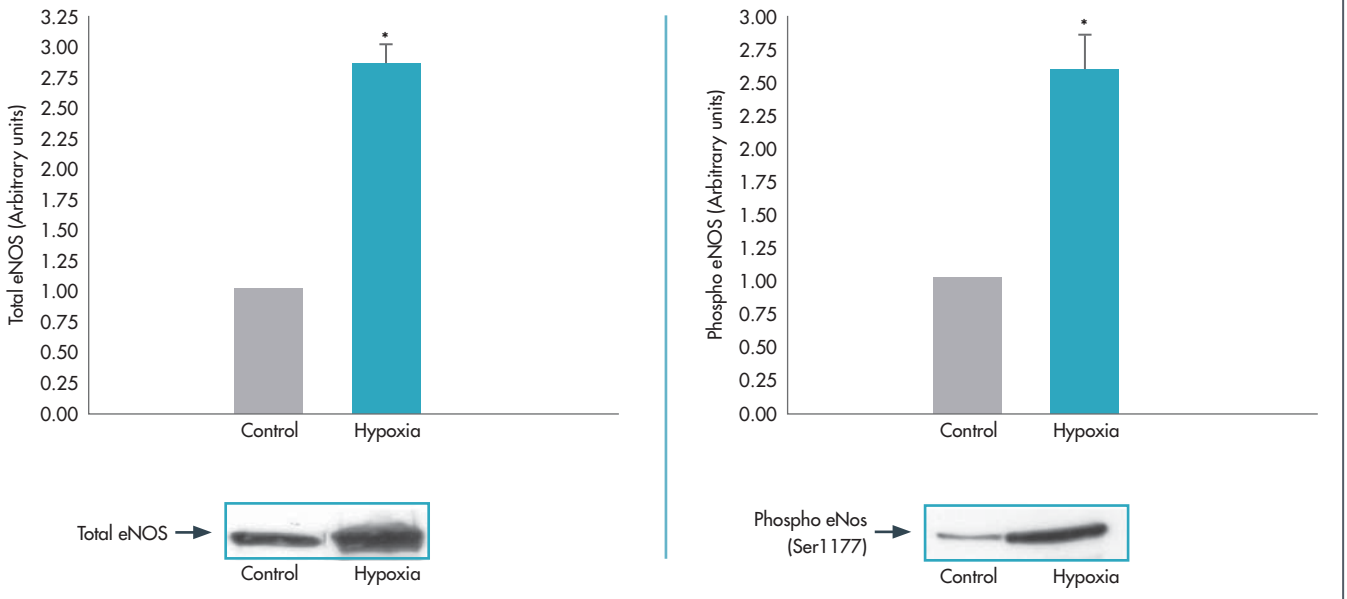


FIGURE 7: Total eNOS protein and activated eNOS (phosphorylated eNOS at Ser 1177) in CMECs exposed to two different hypoxia protocols. In the first protocol, CMECs remained in culture and incubated in low-serum culture medium for 18 h under a low PO_2 atmosphere (O_2: 1%). In the second protocol, CMECs were removed from culture by trypsinisation and the isolated cells were subsequently pelleted and covered with a mineral oil layer (hypoxic pelleting) for 60 min.

A: Western blotting data from cultured cells exposed to 18 h low PO2 incubation showing a 4-fold increase in total eNOS protein expression and 4.9-fold increase in phosphorylated eNOS levels respectively.



B: Western blotting data from isolated CMECs exposed to hypoxic pelleting for 60 min, showing a 2.8-fold increase in total eNOS protein expression and 2.6-fold increase in phosphorylated eNOS levels respectively. *: *$p < 0.05$ vs. control.*

(Blots and graphs reproduced with the permission of Springer Verlag and obtained from: Strijdom H, Friedrich SO, Hattingh S, et al. Hypoxia-induced regulation of nitric oxide synthase in cardiac endothelial cells and myocytes and the role of the PI3-K/PKB pathway. Mol Cell Biochem 2009;321(1-2): 23-35.)

a description of this protocol), total eNOS protein increased by 2.8-fold, and activated levels of eNOS by 2.6-fold[28] (Figure 7B). In this instance, it seems as if the increase in activated eNOS was largely determined by the upregulation of eNOS protein, as the activated/total eNOS ratio was ~ 1. Our findings therefore point to a pivotal role for eNOS in hypoxia, and it seems that the increased NO production in CMECs is mainly derived from eNOS – either due to upregulation of eNOS protein or due to a relative increase in activated eNOS levels (via phosphorylation at the Ser 1177 residue). These results dispel the traditionally held notion that eNOS is predominantly associated with maintenance of physiological function. However, some studies (mainly in endothelial cells of non-cardiac origin) showed opposite effects. As mentioned previously, in saphenous vein and pulmonary artery endothelial cells, hypoxia induced down regulation of eNOS protein and in human umbilical vein endothelial cells, a 40-60% reduction in eNOS mRNA levels was observed[37] suggesting that endothelial cell responses to hypoxia are seemingly determined by their anatomical location, with endothelial cells of cardiac origin generally tending to increase their eNOS protein content and activation and produce larger amounts of NO.

MECHANISMS OF eNOS ACTIVATION IN CMECs

At this point, it was clear that hypoxia induced NO production mainly via eNOS, with apparently no participation from iNOS. However, the upstream mechanism through which eNOS is phosphorylated and therefore activated in CMECs remained unclear.[28] One possibility was that the phosphatidylinositol-3 kinase (PI-3K)/protein kinase B (PKB/Akt) pathway is switched on during hypoxia, with subsequent phosphorylation of eNOS by activated PKB/Akt. Although this eNOS activating mechanism was observed in porcine coronary endothelial cells[30] it had not been described in CMECs before. Therefore, we examined the effect of hypoxia on PKB/Akt, and the findings showed that total PKB/Akt protein expression remained unchanged in hypoxic CMECs compared to normoxic controls; however, activated PKB/Akt (phosphorylated PKB/Akt at Ser 473) increased by 1.7-fold to 3-fold, depending on the hypoxia protocol (Figure 8A). Therefore, in both instances, the relative activated/total PKB/Akt ratio indicated that the increase in activated levels was greater than the increases observed in total protein expression. The fact that activated levels of both eNOS and PKB were significantly increased after identical periods of hypoxia was a promising finding suggestive of a possible mechanistic link between the two. Our hypothesis was further validated when we inhibited the PI-3K – PKB/Akt pathway during hypoxia and observed a significant reduction in NO production compared to untreated hypoxic cells (Figure 8B).

The above data suggest that hypoxia induces increased amounts of NO in CMECs, which are mainly generated by activated eNOS, which seems to be consequential to phosphorylation of its Ser 1177 residue by activated PKB/Akt. However, the molecular mechanism by which hypoxia switches on the PI3-K –PKB/Akt pathway upstream from eNOS is unknown. Interestingly, in separate experiments, we observed a similar PI3-K - PKB/Akt – eNOS – NO mechanism at play in hypoxic cardiomyocytes; however, in the latter cell type we suspect there to be a greater contribution from iNOS than in CMECs, as iNOS protein was expressed in our cardiomyocytes and iNOS-specific inhibition significantly reduced NO production.[26,28] It has to be noted that although eNOS seems to be the major role-player in the production of NO in hypoxic CMECs, inhibition of the enzyme did not completely abolish NO production.[26] As iNOS was not involved in our model of CMECs, and a significant role for nNOS in CMECs has yet to be found, the possibility of a contribution from NOS-independent sources of NO, such as acidosis-induced reduction of nitrites, cannot be excluded.[29]

CONCLUSION

In this review paper, we described the importance of the endothelial cells that line the capillaries of the myocardium. Collectively, these endothelial cells form a unique and functionally distinct subtype, with one factor that distinguishes them from any other endothelial cell subtype in the heart: location. Positioned within 1 µm from the functional units of the heart's contractile machinery, the cardiomyocytes, the CMECs are in an ideal position to influence and regulate myocardial function. This is achieved through the release of a variety of bioactive molecules. Of particular interest, and emphasised in this paper, is the role of CMECs as suppliers of NO, one of the most important chemical messengers in the body. The unique properties of NO as a mediator of paracrine communication are derived from its gaseous and

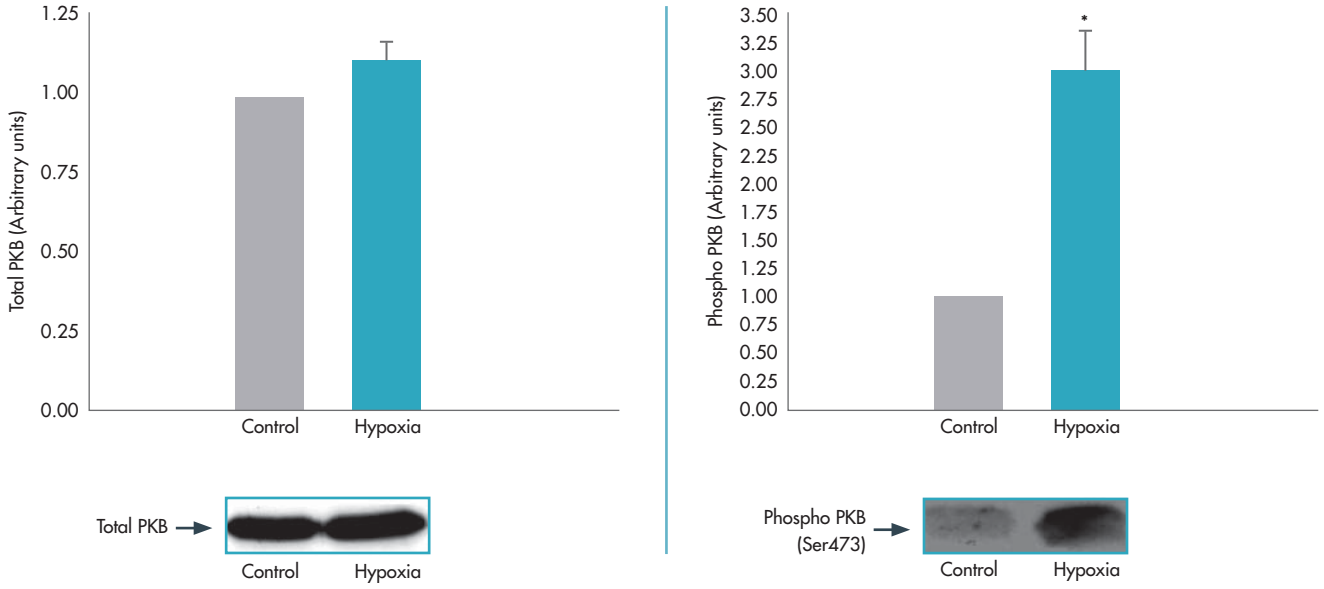
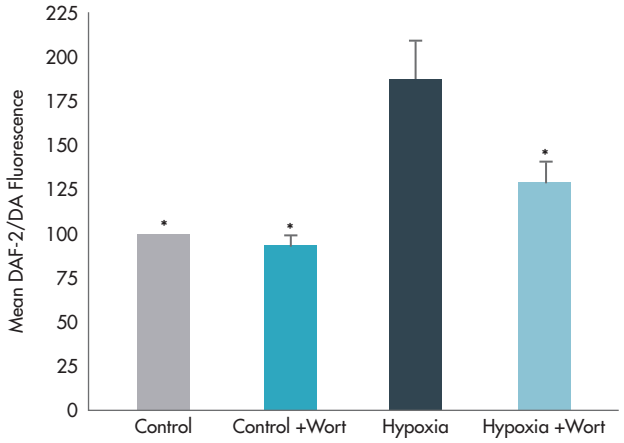


FIGURE 8: Establishing a role for the PI3-K – PKB/Akt pathway in hypoxia-induced eNOS activation.

A: Western blotting data from isolated CMECs exposed to hypoxic pelleting for 60 min, showing unchanged total PKB/Akt protein expression and 3-fold increase in phosphorylated PKB/Akt levels respectively. **: p < 0.05 vs. control.*



B: Bar chart depicting NO production in isolated CMECs exposed to 60 min hypoxic pelleting with or without administration of the PI3-K – PKB/Akt pathway inhibitor, Wortmannin (100 nM). Wortmannin administration resulted in a significant reduction in NO production in hypoxic CMECs. **: p < 0.05 vs. untreated hypoxia.*

(Blots and graphs reproduced with the permission of Springer Verlag and obtained from: Strijdom H, Friedrich SO, Hattingh S, et al. Hypoxia-induced regulation of nitric oxide synthase in cardiac endothelial cells and myocytes and the role of the PI3-K/PKB pathway. Mol Cell Biochem 2009;321(1-2): 23-35.)

free radical nature, allowing it to diffuse freely from one cell to another and exert a high degree of reactivity with a host of target molecules.[27] It is therefore not an exaggeration to describe NO as the ideal chemical messenger! The importance of NO is further underlined by its role as a potent cardio-protective agent, and a plethora of evidence exists showing just how efficient NO is in protecting the myocardium against damage caused by ischaemia, ischaemia-reperfusion and hypoxia.[10,11]

A crucial shortcoming in literature, however, has been the lack of studies that:

- directly and quantitatively measure intracellular production of NO,
- explore the mechanisms underlying NO generation, and
- compare the above findings in CMECs and cardiomyocytes.

In the absence of such knowledge, there is no foundation on which further studies can be built that could ultimately explain, and not merely speculate on, the nature and biological effects of CMEC cardiomyocyte cross-talk, especially with regard to NO as messenger molecule.

In our studies, we attempted to address some of these shortcomings.

We described the interesting relation between CMECs and cardiomyocytes, both known to be NO-generating cell types, with regard to the amount of NO produced by each. Our investigations, the first to directly measure and compare intracellular NO levels in these two cell types on a cell-to-cell basis, validated the previously held assumption that CMECs are indeed greater producers of NO and these data were further supported when it became evident that the eNOS content also followed a similar trend: NO production was ~ 26 times higher per cell and eNOS expression ~ 22 times higher in CMECs per cell compared to cardiomyocytes under normoxic conditions. Furthermore, our findings confirmed existing data in the literature with regard to NO production during hypoxia showing significant increases in the hypoxic cells. In fact, hypoxia managed to further widen the gap between the amount of NO produced in CMECs and cardiomyocytes, suggesting that even more NO is released into the interstitium by hypoxic CMECs. We have also identified the PI-3K - PKB/Akt pathway as a likely upstream activating mechanism of eNOS-derived NO production during hypoxia, described for the first time in CMECs.

In view of our findings and applying the laws of simple diffusion, it is likely that a NO concentration gradient exists from the CMECs to the cardiomyocytes, and that excess CMEC-derived NO may diffuse into cardiomyocytes ("spill-over diffusion") with as yet undetermined effects. These data have important implications for our understanding of the paracrine communication between CMECs and cardiomyocytes in which NO is the messenger. It is generally thought that the paracrine effects of NO released by CMECs are associated with sustaining various physiological functions in cardiomyocytes, such as contractility/relaxation, growth and development and other metabolic functions. However, it may not be as simple as that, since our data suggest there might be a larger NO concentration gradient directed towards the cardiomyocytes than previously thought, particularly under hypoxic conditions. One should also bear in mind that hypoxic cardiomyocytes themselves produce increased amounts of NO, in addition to the CMEC-derived NO that diffuses from the interstitium, which may lead to the accumulation of relatively high concentrations of NO in these cells. Whether the spill-over diffusion of excess NO into the cardiomyocytes is beneficial or harmful, remains unanswered and further investigations are necessary. From previous data, however, it is known that large amounts of NO result in the generation of the potentially cytotoxic radical, peroxynitrite and its downstream derivatives,[23] which should be considered when assessing the impact of spill-over diffusion into the cardiomyocytes.

ACKNOWLEDGEMENTS

This study was sponsored in part by the National Research Foundation of South Africa, the Medical Research Council of South Africa and the Harry Crossley Foundation (University of Stellenbosch).

REFERENCES

1. Münzel T, et al. Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expression of the soluble guanylyl cyclase and the cGMP-dependent protein kinase. Arterioscler Thromb Vasc Biol 2005;25: 1551-1557.
2. Esper RJ, et al. Endothelial dysfunction in normal and abnormal glucose metabolism. Adv Cardiol 2008;45:17-43.
3. Bitar MS, et al. Nitric oxide dynamics and endothelial dysfunction in type II model of genetic diabetes. Eur J Pharmacol 2005;511:53-64.
4. Hsieh PCH, et al. Endothelial-cardiomyocyte interactions in cardiac development and repair. Annu Rev Physiol 2006;68:51-66.
5. Brutsaert DL. Cardiac endothelial-myocardial signalling: its role in cardiac growth, contractile performance, and rhythmicity. Physiol Rev 2003;83:59-115.
6. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. Biochem J 2001;357(Pt 3):593-615.
7. Singh S, Evans TW. Nitric oxide, the biological mediator of the decade: fact or fiction? Eur Respir J 1997;10: 699-707.
8. Bath PM, Hassall DG, Gladwin AM, et al. Nitric oxide and prostacyclin. Divergence of inhibitory effects on monocyte chemotaxis and adhesion to endothelium in vitro. Arterioscler Thromb 1991;11(2):254-60.
9. Marui N, Offermann MK, Swerlick R, et al. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J Clin Invest 1993;92(4):1866-1874.
10. Bolli R. Cardio-protective function of inducible nitric oxide synthase and role of nitric oxide in myocardial ischaemia and preconditioning: an overview of a decade of research. J Mol Cell Cardiol 2001;33:1897-1918.
11. Jones SP, Bolli R. The ubiquitous role of nitric oxide in cardio-protection. J Mol Cell Cardiol 2006;40:16-23.
12. Shah AM, MacCarthy PA. Paracrine and autocrine effects of nitric oxide on myocardial function. Pharmacol Ther 2000;86(1):49-86.
13. Brutsaert DL, Franken P, Andries LJ, et al. Cardiac endothelium and myocardial function. Cardiovasc Res 1998;38:281-290.
14. Linask KK, Lash JW. Early heart development: dynamics of endocardial cell sorting suggests a common origin with cardiomyocytes. Dev Dyn 1993;196(1):62-9.
15. Marchionni MA. Cell-cell signalling. neu-tack on neuregulin. Nature 1995;378(6555): 334-5.
16. Korecky B, Hai CM, Rakusan K. Functional capillary density in normal and transplanted rat hearts. Can J Physiol Pharmacol 1982;60:23-32.
17. Ziolo MT, Kohr MJ, Wang H. Nitric oxide signalling and the regulation of myocardial function. J Mol Cell Cardiol 2008;45(5):625-32.
18. Bredt DS. Nitric oxide signalling specificty – the heart of the problem. J Cell Sci 2003;116:9-15.
19. O'Donnell VB, Eiserich JP, Chumley PH, et al. Nitration of unsaturated fatty acids by nitric oxide-derived reactive nitrogen species peroxynitrite, nitrous acid, nitrogen dioxide, and nitronium ion. Chem Res Toxicol 1999;12:83-92.
20. Balligand JL, Cannon PJ. Nitric oxide synthases and cardiac muscle. Autocrine and paracrine influences. Arterioscler Thromb Vasc Biol 1997;17(10):1846-58.
21. Schulz R, Kelm M, Heusch G. Nitric oxide in myocardial ischaemia/reperfusion injury. Cardiovasc Res 2004;61:402-413.
22. Andries LJ, Brutsaert DL, Sys SU. Non-uniformity of endothelial nitric oxide synthase distribution in cardiac endothelium. Circ Res 1998;82(2):195-203.
23. Ferdinandy P, Schulz R. Nitric oxide, superoxide, and peroxynitrite in myocardial ischaemia-reperfusion injury and preconditioning. Br J Pharmacol 2003;138: 532-543.
24. Lange M, Enkhbataar P, Nakano Y, et al. Role of nitric oxide in shock: the large animal perspective. Front Biosci 2009;14:1979-1989.
25. Strijdom H, Muller C, Lochner A. Direct intracellular nitric oxide (NO) detection in isolated adult cardiomyocytes: Flow cytometric analysis using the fluorescent probe, diaminofluorescein (DAF). J Mol Cell Cardiol 2004;37:897-902.
26. Strijdom H, Jacobs S, Hattingh S, et al. Nitric oxide production is higher in rat cardiac micro vessel endothelial cells than ventricular cardiomyocytes in baseline and hypoxic conditions: a comparative study. FASEB J 2006;20:314-316.
27. Mount PF, Kemp BE, Power DA. Regulation of endothelial and myocardial NO synthesis by multi-site eNOS phosphorylation. J Mol Cell Cardiol 2007;42: 271- 279.
28. Strijdom H, Friedrich SO, Hattingh S, et al. Hypoxia-induced regulation of nitric oxide synthase in cardiac endothelial cells and myocytes and the role of the PI3-K/PKB pathway. Mol Cell Biochem 2009;321(1-2):23-35.
29. Millar TM, Stevens CR, Benjamin N, et al. Xanthine oxidoreductase catalyzes the reduction of nitrates and nitrite to nitric oxide under hypoxic conditions. FEBS Lett 1998;427:225-228.
30. Chen JX, Meyrick B. Hypoxia increases Hsp90 binding to eNOS via PI3-KAkt in porcine coronary artery endothelium. Lab Invest 2004;84:182-190.
31. Xu XP, Pollock JS, Tanner MA, et al. Hypoxia activates nitric oxide synthase and stimulates nitric oxide production in porcine coronary resistance arteriolar endothelial cells. Cardiovasc Res 1995;30(6):841-7.
32. Justice JM, Tanner MA, Myers PR. Endothelial cell regulation of nitric oxide production during hypoxia in coronary micro vessels and epicardial arteries. J Cell Physiol 2000;182:359-365.
33. Takemoto M, Sun J, Hiroki J, et al. Rho-kinase mediates hypoxia-induced down regulation of endothelial nitric oxide synthase. Circulation 2002;106(1):57-62.
34. Jung F, Palmer, Zhou N, et al. Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. Circ Res 2000;86: 319-325.
35. Massion PB, Feron O, Dessy C, et al. Nitric oxide and cardiac function: Ten years after, and continuing. Circ Res 2003;93:388-398.
36. Dimmeler S, Fleming I, Fisslthaler B, et al. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. Nature 1999;399:601-605.
37. McQuillan LP, Leung GK, Marsden PA, et al. Hypoxia inhibits expression of eNOS via transcriptional and posttranscriptional mechanisms. Am J Physiol 1994; 5:1921-H1927.