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Endothelin-1 and Phenylephrine-induced Activation of the Phosphoinositide Cycle Increases Cell Injury of Cultured Cardiomyocytes Exposed to Hypoxia/ Reoxygenation

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H. A. A. VAN HEUGTEN, K. BEZSTAROSTI and J. M. J. LAMERS. Endothelin-1 and Phenylephrine-induced Activation of the Phosphoinositide Cycle Increases Cell Injury of Cultured Cardiomyocytes Exposed to Hypoxia/Reoxygenation. *Journal of Molecular and Cellular Cardiology* (1994) **26**, 1513–1524. We explored the effect of glucose-free hypoxia/ reoxygenation of cultured neonatal rat ventricular myocytes on endothelin-1 and α_1-adrenoceptor induced activity of the phosphoinositide cycle. At the same time the influence of these agonists on depletion of energy-rich phosphates and cellular damage was assessed. Glucose-free hypoxia did not lead to an increase in basal phospholipase C activity. However, endothelin-1 (10^{-8}M) and phenylephrine (10^{-5}M) evoked activation of phospholipase C was attenuated after 60 min of hypoxia and declined to 38% and 30% respectively of normoxic values after 90 min of hypoxia. During glucose-free hypoxia, phosphatidylinositol 4,5-bisphosphate, the substrate for phospholipase C, but not phosphatidylinositol or phosphatidylinositol 4-monophosphate was seen to decline to 59% of normoxic values which was independent of activation of phospholipase C by agonists. ATP levels decreased after 30 min of hypoxia and declined to 29% relative to normoxic control after 90 min of hypoxia. Total adenine nucleotide levels showed a similar pattern. The presence of 10^{-8}M endothelin-1 during hypoxia did not influence the magnitude of ATP depletion. However, after 15 min of reoxygenation, by itself not significantly leading to recovery of ATP levels, ATP levels were decreased by endothelin-1 as compared to hypoxia/reoxygenation without phospholipase C agonist. Cellular damage as determined by lactate dehydrogenase leakage was not observed during 90 min hypoxia. Reoxygenation resulted in a three-fold increase in enzyme release relative to normoxic control. In the presence of endothelin-1 or phenylephrine this reoxygenation-induced damage was respectively 1.7 and 3.0-fold increased. We conclude that the agonist-induced activity of the phosphoinositide cycle is decreased in time during glucose-free hypoxia, partially through a decrease in phosphatidylinositol 4,5-bisphosphate level. However, the remaining activity may give rise to increased cellular damage. As endothelin-1 and α_1-adrenergic amines are known to be released during myocardial ischemia, stimulation of the phosphoinositide cycle by these agonists might be an important factor in determining the magnitude of myocardial injury.

KEY WORDS: Endothelin-1; α_1-adrenergic agonist; Cardiomyocyte; Hypoxia; Reoxygenation; Inositol-phosphates; Phosphatidylinositol 4,5-bisphosphate; Energy metabolism; Cell injury

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Introduction

Endothelin-1 (ET-1) is an endothelium-derived 21-residue peptide, initially discovered to be synthesized and secreted by endothelial cells (Yanagisawa *et al.*, 1988) but that was recently described to be produced in and secreted by cardiac myocytes (Suzuki *et al.*, 1993) as well. ET-1 has an extremely potent and sustained vasoconstrictor effect (Yanagisawa *et al.*, 1988). ET-1 also has both positive inotropic and chronotropic effects on the heart (Moravec *et al.*, 1989) and was reported to induce cardiac hypertrophy (Ito *et al.*, 1991). The release of ET is increased during hypoxia and myocardial infarction leads to increased plasma levels of ET (Yasuda *et al.*, 1990). Moreover, hypoxia increases the number of ET-1 binding sites (Liu *et al.*, 1990). Myocardial hypoxia and/or ischemia has also been shown to enhance adrenergic neural traffic, to release endogenous catecholamines from nerve terminals and to increase myocardial responsiveness to α- and β-adrenergic stimulation. Hypoxia-induced increase of α_1-adrenergic receptors is accompanied by an initial increase in phospholipase C (PLC) activity in response to receptor stimulation (Heathers *et al.*, 1989; Kagiya *et al.*, 1991).

The signalling pathway activated by ET-1 and α_1-adrenergic agonist involves activation of PLC (Prasad *et al.*, 1991; Lamers *et al.*, 1992; Heugten, van *et al.*, 1993) resulting in long lasting accumulation of inositol phosphates ($InsP_n$) which only in the case of ET-1 is desensitized in time leading to sustained signal transduction at a lower level (Heugten, van *et al.*, 1993). Inositol 1,4,5-trisphosphate, produced after occupation of α_1-adrenergic and ET-1 receptors, acts as an intracellular second messenger by inducing intracellular Ca^{2+} mobilization from the sarcoplasmic reticulum (Vigne *et al.*, 1990; Gambassi *et al.*, 1992). Diacylglycerol, also produced by cleavage of phosphatidylinositol 4,5-bisphosphate [PtdIns (4, 5)P_2] activates protein kinase C (PKC). For instance, it was shown that PKC was activated by ET-1 (Bogoyevitch *et al.*, 1993) as well as by phenylephrine (PHE) (Henrich and Simpson, 1988). Activation of PKC by phorbol esters was shown to aggravate hypoxic myocardial injury presumably by stimulation of Na^+/H^+ exchange leading to Ca^{2+} overload by Na^+/Ca^{2+} exchange (Ikeda *et al.*, 1988). A secondary effect of activation of PKC by ET-1 is enhanced Ca^{2+} entry through the T-type Ca^{2+} channels (Furukawa *et al.*, 1992). Therefore, it is likely that the activation of the phosphoinositide cycle by α_1-adrenoceptors and/or ET-1 during myocardial hypoxia and/or ischemia contributes to aggravation of the development of cellular Ca^{2+} overload.

The possibility that a long lasting high phosphoinositide turnover rate through increased ET-1 and α_1-adrenergic amine levels during hypoxia leads to increased hypoxia/reoxygenation injury prompted us to investigate the effect of ET-1 and PHE on normoxic cultured cardiomyocytes as well as in cardiomyocytes exposed to glucose-free hypoxia/reoxygenation, taking enzyme leakage and ATP depletion as indicators of cellular injury and derangement of energy metabolism respectively.

Materials and Methods

Cell culture

Primary cultures of neonatal rat ventricular myocytes were prepared from 1–2 day-old Wistar rats as described before (Yagev *et al.*, 1984) using preplating (Blondel *et al.*, 1971) to further increase cardiomyocyte to non-myocyte ratio. Cells were seeded at 150 to 175×10^3 cells/cm^2 and cultures contained about 90% cardiomyocytes as judged by glycogen staining. Cardiomyocytes were grown at 37°C and 5% CO_2 in DMEM/M199 (4:1) containing 6 mM glucose, 5% fetal calf serum and 5% horse serum. Experiments were routinely performed 5 or 6 days after plating of the cells.

Hypoxia/reoxygenation protocol

Cardiomyocytes, grown in 24-well plates (Costar, Cambridge, MA, USA) were washed with incubation buffer (130 mM NaCl; 4.7 mM KCl; 1.3 mM $CaCl_2$, 0.44 mM NaH_2PO_4, 1.1 mM $MgSO_4$; 20 mM $NaHCO_3$; 20 mM HEPES; pH 7.4, 37°C and aerated with 95% air/5% CO_2) with or without 6 mM glucose. After removing the buffer, cells for hypoxia/reoxygenation experiments were transferred to the heated floor (37°C) of a small airtight incubator chamber pregassed with 95% N_2 and 5% CO_2 where hypoxic incubation buffer (total volume 500 μl, pregassed with 95% N_2 and 5% CO_2) was added. The chamber was continuously gassed with N_2/CO_2 and pO_2 was monitored with a Servomex oxygen analyzer and was found to be lower than 1.0% during the hypoxic period. For reoxygenation, the hypoxic incubation buffer was replaced by 500 μl normoxic buffer and the chamber was gassed with 95% air/5% CO_2. After 90 min of normoxia, buffer was changed to mimic the reoxygenation protocol.

In a separate series of experiments, reoxygenation was achieved by replacing 95% N_2/5% CO_2 with 95% air/5% CO_2 without buffer change. In all hypoxic experiments solutions to be added at the start of or during hypoxia were preëquilibrated in the hypoxic chamber for at least 2 h.

Measurement of water-soluble inositolphosphates

Cardiomyocytes were labelled with 2 μCi *myo*-[2-^{3}H]inositol/ml for 24 h in complete growth medium. Prior to performing the experiments, myocytes were washed in incubation buffer and the normoxia and hypoxia/reoxygenation protocol was performed as described above. For stimulation of the cells with agonist, ET-1 (10^{-8} M endconcentration) or PHE (10^{-5} M) were added to the incubation buffer without buffer change, both in the presence of 10 mM LiCl to inhibit inositolphosphatase activity. These agonist concentrations were previously shown to give near-maximal activation of PLC (Heugten, van *et al.*, 1993). Incubations were terminated by rapidly washing the cells with ice-cold buffer followed by two successive extractions with 250 μl ice-cold 4% $HClO_4$ after which lipids were extracted twice with 250 μl ice-cold CH_3OH:HCl (100:1 v/v). The perchloric acid extract was rapidly neutralized by addition of 100 μl 2 M KOH; 1 M K_2CO_3. The water-soluble inositol phosphates in the perchloric acid extract were quantified by chromatography on Dowex AG 1-X8 as originally described by Berridge *et al.* (1982). Amounts of $InsP_n$ are expressed as percentage of the total cellular amount of [2-^{3}H]-inositol labelled products defined as the sum of water-soluble inositol-containing products and inositol-containing lipids.

Measurement of inositol-containing lipids

Lipids were extracted from the cardiomyocytes with CH_3OH:HCl (100:1 v/v) as described above. One volume of $CHCl_3$ and 0.5 vol of 2.5 M HCl were added to this extract resulting in phase-separation. The organic, lower phase was reextracted once with one volume of $CHCl_3$:CH_3OH:0.6 M HCl (3:48:47 v/v/v). Phosphatidylinositol (PtdIns), phosphatidylinositol 4-monophosphate [PtdIns(4)P] and PtdIns(4,5)$_2$ in the organic phase were analysed by thin layer chromatography and quantified as described before (Heugten, van *et al.*, 1993). Again, PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 are expressed as percentage of total cellular [2-^{3}H]inositol.

Measurement of adenine nucleotides

After normoxic or hypoxic incubation in the presence or absence of agonists without LiCl, buffer was removed and stored on ice for determination of enzyme leakage. The cardiomyocytes were then extracted with 250 μl ice-cold 4% $HClO_4$ in 175 mM H_3PO_4 for 2 min after which the extract was removed from the well and neutralized with 58 μl 2 M KOH; 1 M K_2CO_3. High performance liquid chromatography of 100 μl extract was performed on a reverse phase C-18 column (Beckman) as described before (Sellevold *et al.*, 1986; Sassen *et al.*, 1991), except that 175 mM KH_2PO_4; 2.3 mM tetrabutylammonium hydrogensulphate; 2.5% acetonitrile; pH 6.25 was used as running buffer. The content of adenine nucleotides, expressed as μmol/mg cellular protein was calculated by peak-integration and comparison with standards of known quantity.

Determination of lactate dehydrogenase activity

After normoxic or hypoxic incubation, the buffer was collected and stored on ice for determination of lactate dehydrogenase (LDH) activity. Enzymatic activity was assayed in 95 mM potassium phosphate buffer (KH_2PO_4; K_2HPO_4); 7.6 mM sodium pyruvate; 0.2 mM NADH; pH 7.0 by measuring the decrease in absorption at 340 nm (for NADH; $\varepsilon_{340} = 6.22\,cm^2/\mu mol$) and is expressed in mUnits/mg cellular protein (1 Unit = 1 μmol pyruvate converted into lactate/min).

Determination of total cellular protein

After extracting the cardiomyocytes for determination of adenine nucelotides as described above, total cellular protein was dissolved by adding 250 μl 1 M NaOH to the well and incubating overnight at 4°C. Hereafter, the dissolved protein in 1 M NaOH was removed from the well and used for protein determination according to Bradford (1976) using bovine serum albumin in 1 M NaOH as standard. Cardiomyocytes in one well ($250–300 \times 10^3$ cells) contained about 100 μg of protein.

Statistical analysis

Data were evaluated for statistical significance using analysis of variance, the Student-Newman-Keuls test and the Mann-Whitney rank sum test.

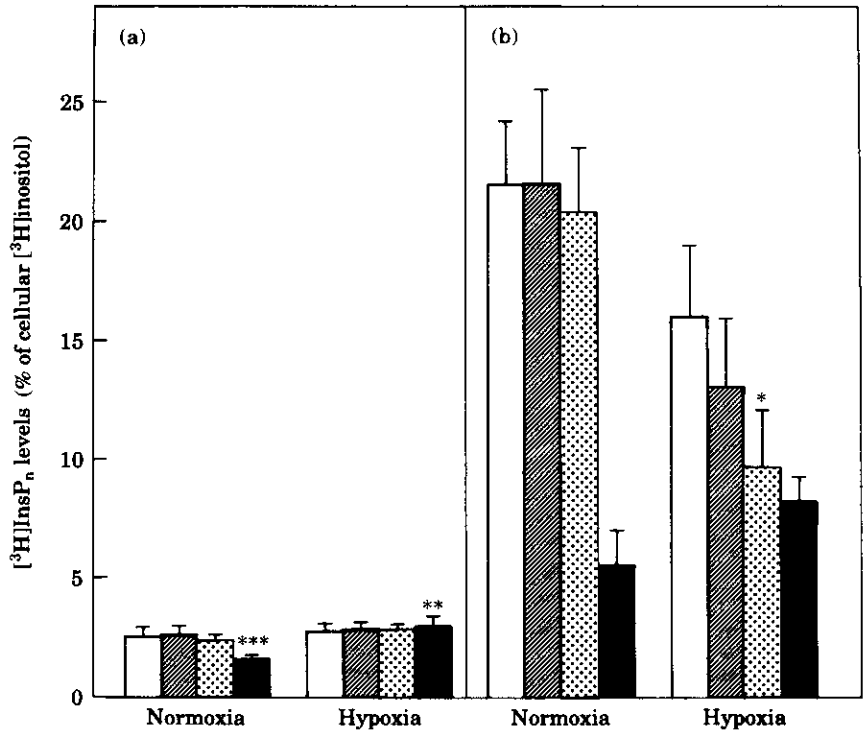

Figure 1 ET-1 induced $InsP_n$ accumulation during normoxia or hypoxia and subsequent reoxygenation. Cardiomyocytes, prelabelled with *myo*-[2-^{3}H]inositol, were incubated with buffer under normoxic or hypoxic conditions as described in the experimental section. Normoxia and hypoxia was performed during 30 (□), 60 (▨) and 90 (▩) min, while incubation in normoxic buffer after 90 min hypoxia or normoxia lasted 15 min (■). Hereafter, cells were challenged with LiCl (10 mM) in the absence (a) or presence (b) of ET-1 (10^{-8} M). Incubation in the presence of Li^+ was always for 15 min. After incubation, the cells were extracted and $InsP_n$ was quantified. Further details are described in Materials and Methods. Results are mean ± S.E.M. for 7 to 10 experiments. *P<0.02 and **P<0.005 *v* normoxia; ***P<0.02 *v* 90 min normoxia.

Results

To evaluate the ET-1 evoked signal transduction during glucose-free hypoxia/reoxygenation and possible effects of ET-1 on hypoxia/reoxygenation-induced cellular injury, we established a model of cultured neonatal rat ventricular myocytes exposed to 95% N_2/5% CO_2 (hypoxia) for periods up to 90 min followed by 15 min reoxygenation by buffer change in 95% air/5% CO_2. The severity of hypoxia/reoxygenation was characterized by monitoring ATP depletion and enzyme release as will be described below. As mentioned before, ET-1 is a potent activator of the phosphoinositide cycle (Heugten, van *et al.*, 1993). Incubation of cardiomyocytes in normoxic buffer for 90 min did not change the ET-1 (10^{-8} M) induced $InsP_n$ accumulation during a 15 min incubation following exposure to the buffer [Fig. 1(b)]. Surprisingly however, a simple buffer change after 90 min normoxic incubation strongly attenuated the ET-1 evoked (22% of the value after 90 min normoxia) as well as the basal (to 68%) PLC activity. Hypoxia *per se* did not result in activation of PLC while reoxygenation after hypoxia by buffer change did not lead to the attenuation of the basal PLC activity that was seen after normoxia [Fig 1(a)]. Most important however, stimulation for 15 min with 10^{-8} M ET-1 after 30, 60 and 90 min hypoxia showed a gradual decline in agonist-induced $InsP_n$ accumulation to 38% [Fig 1(b)]. Reoxygenation (including a change of buffer) for 15 min after hypoxia followed by 15 min stimulation with 10^{-8} M ET-1 showed that agonist-dependent $InsP_n$ accumulation returned to the level found after buffer change during normoxia. In order to determine whether the buffer change-induced reduction in ET-1 evoked PLC activity had any effect on the influence of reoxygenation, another series of experiments were performed where reoxygenation was accomplished by placing the cells in 95% air/5% CO_2. No significant difference in PLC activity after ET-1 stimulation was noticed when cardiomyocytes where exposed to normoxia for either 90 min or 90 + 15 min. Again, ET-1 evoked PLC

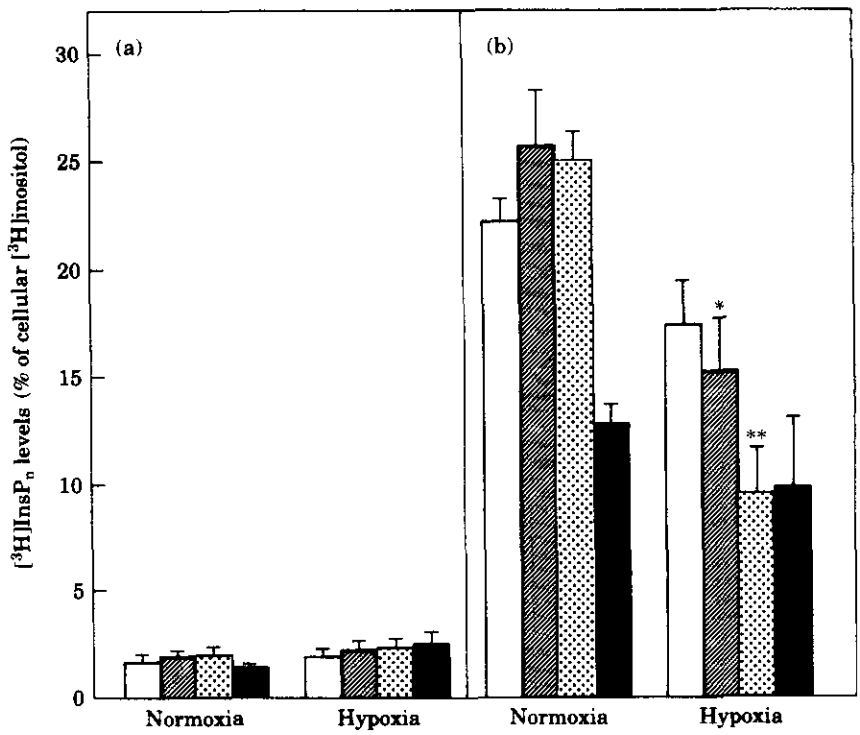

Figure 2 Phenylephrine induced $InsP_n$ accumulated during normoxia or hypoxia and subsequent reoxygenation. Cardiomyocytes were exposed to normoxia or hypoxia/reoxygenation as described in the legends to Figure 1 and exposed to 10^{-5}M PHE (b) or vehicle (a) in the presence of 10mM LiCl. $InsP_n$ levels were quantified as described in Materials and Methods. Results are mean ± S.E.M., $n=4$. *$P<0.05$ and **$P<0.005$ *v* normoxia.

activity was decreased after 90min of hypoxia (to 31 ± 9%, $n=4$, relative to normoxic control, 90min). Reoxygenation, now accomplished without buffer change, led to complete recovery of stimulatability (121 ± 8%, $n=4$, relative to control, 90 + 15min).

It might be argued that the omission of glucose from the buffer was contributing to the decreased ET-1 evoked PLC activity after 90min hypoxia. However, 90min of hypoxia in the presence of glucose also resulted in attenuated ET-1 evoked PLC activity (26 ± 4% of normoxic control, mean ± range/2 for two independent experiments) indicating that hypoxia *per se* is the major factor reducing PI cycle activity.

To evaluate whether the hypoxia-induced decrease in PLC stimulatability is agonist-dependent we repeated the experiments, now with the α_1-adrenergic agonist PHE (10^{-5}M), also known to be able to activate PLC (Heugten, van *et al.*, 1993). Figure 2(b) shows that hypoxia also produced a reduction in stimulatability of cardiomyocytes with PHE. This result indicates that glucose-free hypoxia induces either a desensitization of cardiomyocytes towards agonists coupled to the phosphoinositide cycle or induces a defect in the operation of the receptor- G-protein -PLC complex. Figure 3(a) shows that part of this attenuated PLC activity might be ascribed to a hypoxia-induced decrease in the level of $PtdIns(4,5)P_2$ which is the substrate for PLC. Reoxygenation did not lead to recovery of the $PtdIns(4,5)P_2$ levels. Stimulation of PLC with ET-1 after normoxic or hypoxic periods did not lead to significant alteration in $PtdIns(4,5)P_2$ levels as compared to unstimulated cardiomyocytes [compare Figs 3(a) and (b)], an observation that correlates well with the rapid resynthesis of PtdIns(4,5)P_2 after agonist-induced activation of PLC as reported before (Heugten, van *et al.*, 1993). Table 1 shows that PtdIns was not significantly altered after hypoxia as was also true for PtdIns(4)P (not shown).

Having shown that hypoxia alters signal transduction through the phosphoinositide pathway, we proceeded to study the effects of persistent activation of this pathway on derangement of energy metabolism and cellular injury induced by hypoxia and reoxygenation. When cardiomyocytes were maintained in buffer with glucose, 90min of hypoxia only slightly affected ATP levels of the cardiomyocytes (Table 2). On the other hand, when cardiomyocytes were exposed to hypoxia in buffer without glucose,

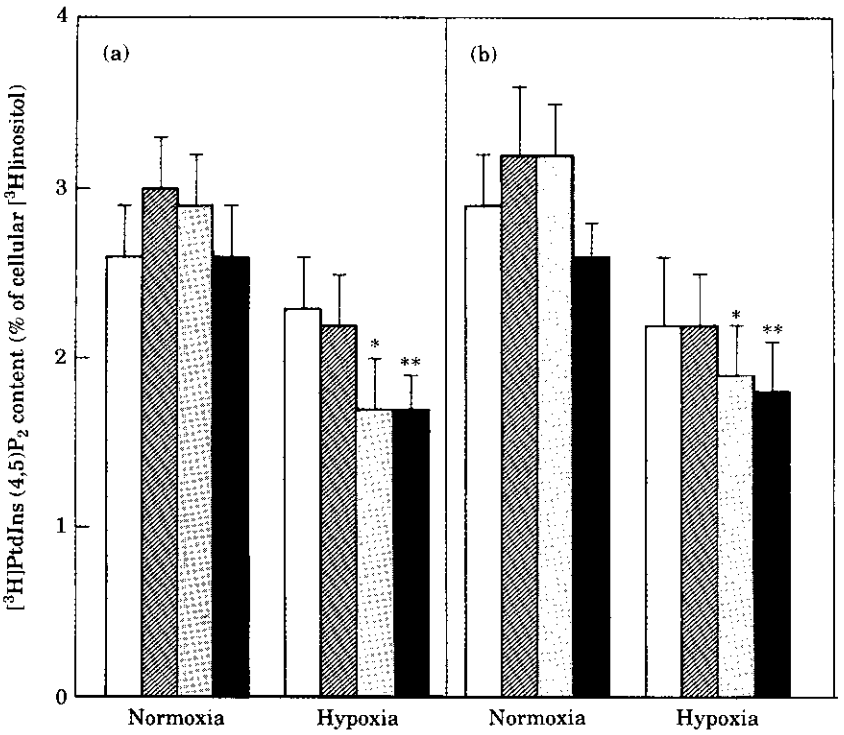

Figure 3 Changes in PtdIns(4,5)P_2 levels during normoxia or hypoxia and subsequent reoxygenation in the absence or presence of ET-1. Cardiomyocytes, prelabelled with *myo*-[2-^{3}H]inositol, were incubated with buffer under normoxic or hypoxia/reoxygenation conditions as described in the legends to Figure 1. Hereafter, cells were incubated with 10^{-8} M ET-1 (b) or vehicle (a), both in the presence of 10 mM LiCl for 15 min. After incubation, $InsP_n$ was extracted followed by lipid extraction as described in the experimental section. Inositol-containing lipids were analysed by thin-layer chromatography and quantified as described before (Heugten, van *et al.*, 1993). Results are mean ± S.E.M., $n=6$. *$P<0.02$ and **$P<0.05$ *v* normoxia.

Table 1 [^{3}H]PtdIns content of cardiomyocytes exposed to normoxia or hypoxia and reoxygenation in the absence or presence of 10^{-8} M ET-1. PtdIns levels during normoxia and hypoxia/reoxygenation were determined in cellular extracts obtained from the experiments described in Figure 3. [^{3}H]PtdIns levels, expressed as percentage of total cellular [^{3}H]inositol are mean ± S.E.M. for six experiments. No significant differences between either normoxia and hypoxia or control versus ET-1 stimulated cells were observed.

	control		+ET-1	
	normoxia	hypoxia	normoxia	hypoxia
30 min	66±4	64±4	60±2	60±3
60 min	68±3	67±3	62±1	67±3
90 min	70±3	65±3	67±3	68±2
90 min + 15 min O_2	74±3	66±3	70±2	67±2

Table 2 Influence of glucose on changes in ATP levels during normoxia or hypoxia in the absence or presence of ET-1. Cardiomyocytes were incubated with buffer without LiCl under normoxic or hypoxic conditions for the indicated periods. During the entire incubation period 10^{-8} M ET-1 was either present or absent. After the incubation adenine nucleotides were extracted and analysed by HPLC as described in the experimental section. Results are mean ± range/2, for two independent experiments and are expressed as percentage of the ATP content after 15 min incubation in normoxic buffer without ET-1 (34.1 and 41.3 μmol/mg protein for two independent experiments respectively).

	control		+ET-1	
	normoxia	hypoxia	normoxia	hypoxia
15 min	100	83±4	87±7	90±3
30 min	99±1	88±2	87±4	92±2
60 min	98±6	88±2	99±1	87±5
90 min	97±2	86±7	92±2	92±11

the ATP levels decreased steadily (Fig. 4). ATP depletion was almost linear in time and was slightly but not significantly reversed during 15 min of reoxygenation (Fig. 4). In the experiments described above reoxygenation was accomplished by buffer change resulting in removal of degradation products of ATP (e.g. hypoxanthine) that might be important for resynthesis of adenine nucleotides. Therefore, experiments were also performed where reoxygenation was brought about without buffer change as described above. After 90 min of hypoxia

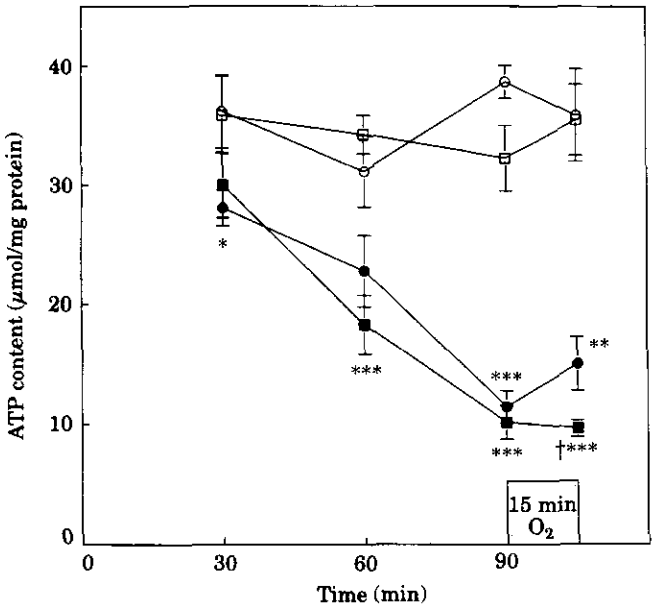

Figure 4 Changes in ATP levels during normoxia or hypoxia and reoxygenation in the absence or presence of ET-1. Cardiomyocytes were incubated with buffer without LiCl under normoxic (open symbols) or hypoxic conditions (closed symbols) for 30, 60 or 90 min. Incubation in normoxic buffer was performed during 15 min succeeding 90 min of hypoxia or normoxia. During the entire incubation period 10^{-8} M ET-1 was either present (□ ■) or absent (○ ●). After the incubation adenine nucleotides were extracted and analyzed by HPLC as described in the experimental section. Results are mean ± S.E.M., $n=5$. †$P<0.05$ *v* minus ET-1 and *$P<0.05$; **$P<0.002$; ***$P<0.001$ *v* normoxia.

ATP levels were decreased to 24 ± 13% and subsequent reoxygenation (15 min) led to partial recovery of ATP levels to 41 ± 12% relative to normoxic control (mean ± range/2 for two independent experiments). Prolonged reoxygenation (60 min) increased ATP levels to 48 ± 2% but still longer reoxygenation (120 min) showed no further increase. These results correlate nicely with earlier reports (Vemuri *et al.*, 1989; Fantini *et al.*, 1990).

To evaluate the effect of PLC activation on glucose-free hypoxia-induced energy depletion, cardiomyocytes were incubated with ET-1 (10^{-8} M) during the entire normoxic or hypoxia/reoxygenation period. This activation did neither significantly alter the ATP levels during normoxia nor the magnitude of ATP depletion during 90 min of hypoxia. However, in the presence of ET-1 reoxygenation for 15 min led to significantly lower levels of ATP. Not only ATP levels were seen to be decreased by glucose-free hypoxia, ADP as well as AMP were decreasing in parallel with ATP (data not shown), resulting in a decrease in total cellular adenine nucleotide levels during hypoxia as shown in Figure 5. Again, the presence of ET-1 resulted in significantly lower total cellular adenine nucleotide levels after 15 min of reoxygenation (Fig. 5).

Irreversible cell injury due to hypoxia, as reflected by enzyme release, is known to increase with prolonged hypoxic periods and is most prominent after reoxygenation (Fantini *et al.*, 1990) that follows short periods (less than 2 h) of hypoxia. Hypoxia/reoxygenation in the presence of glucose did not lead to cellular injury as determined by LDH leakage (results not shown). Figure 6 shows that during normoxia lactate dehydrogenase (LDH) leakage only occurred after buffer changes, the amount released corresponding to about 2.5% of total LDH activity in these cells (not shown). Neither glucose-free hypoxia nor activation of PLC by ET-1 resulted in increased enzyme release. However, when cells were reoxygenated for 15 min after 90 min of glucose-free hypoxia, LDH leakage increased about three-fold as compared to buffer change during normoxia [Fig. 6(a)]. The reoxygenation-induced cell injury was further increased when ET-1 was present during glucose-free hypoxia and reoxygenation [compare Fig. 6(a) and (b)]. A stimulation of hypoxia/reoxygenation-induced LDH leakage by ET-1 was consistently observed and ranged 1.5 to 2.1-fold (1.7 ± 0.2; $n=4$). Although considerable cell batch-to-batch variability in enzyme leakage data was observed (range 118–482 mU LDH/mg protein after reoxygenation minus ET-1 and 150 to 884 mU LDH/mg protein after reoxygenation plus ET-1) statistical significance of difference between the absolute LDH leakage values was obtained ($P<0.05$ by the Mann-Whitney rank sum test). Experiments (not shown)

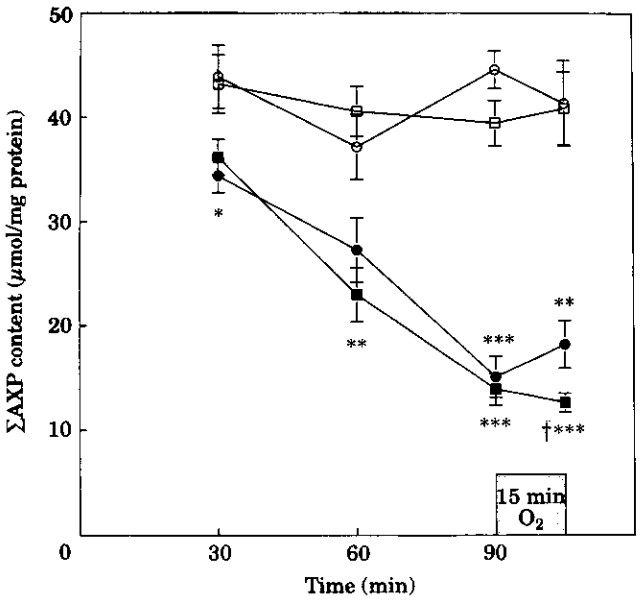

Figure 5 Changes in adenine nucleotide levels during normoxia or hypoxia and subsequent reoxygenation with or without ET-1. Adenine nucleotide levels (sum of ATP, ADP and AMP) were determined in cellular extracts of the experiments described in Figure 4. Open symbols depict normoxia, closed symbols represent hypoxia and subsequent reoxygenation for 15 min. ○ ●; unstimulated cells and □ ■; cardiomyocytes stimulated with 10^{-8} M ET-1. Results are mean ± S.E.M., $n = 5$. †$P = 0.05$ *v* minus ET-1 and *$P < 0.05$; **$P < 0.002$; ***$P < 0.001$ *v* normoxia.

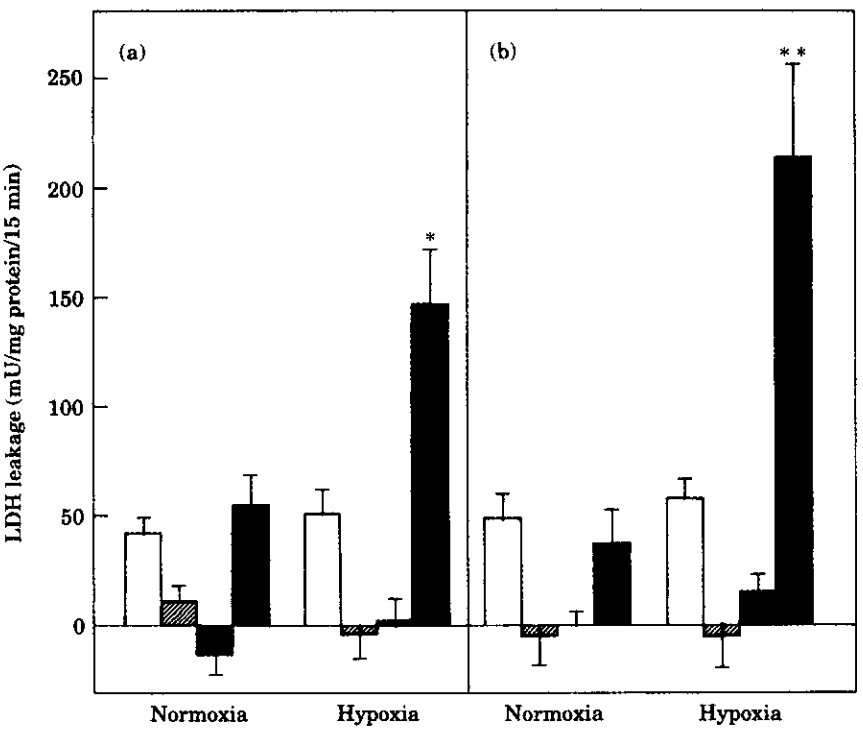

Figure 6 The effect of ET-1 on LDH release during normoxia or hypoxia and subsequent reoxygenation. Cardiomyocytes were subjected to normoxia or hypoxia/reoxygenation as described in the legend to Figures 1 and 4. During the entire incubation period 10^{-8} M ET-1 was either present (b) or absent (a). After the incubation, the buffer was collected and LDH release was determined as described in the experimental section. Results are mean ± S.E.M, $n = 4$–5. *$P < 0.02$ and **$P < 0.001$ *v* buffer change after normoxia, and **$P < 0.05$ *v* hypoxia without ET-1.

to access stimulation of hypoxia/reoxygenation induced cell injury by agonists were also carried out in the presence of 10^{-5}M PHE, again showing stimulation of glucose-free hypoxia/reoxygenation induced LDH leakage (3.0 ± 0.8-fold, $n=4$, $P<0.05$).

Discussion

In this study we investigated the effect of hypoxia/reoxygenation on ET-1 and PHE-induced activation of the phosphoinositide cycle at the same time studying the effect of the presence of ET-1 and PHE on hypoxia/reoxygenation induced derangement of cellular energy metabolism and cell injury. As the presence of glucose in the experiments led to an absence of hypoxia-induced cell injury, we employed glucose-free hypoxia/reoxygenation. We showed that glucose-free hypoxia decreases the phosphoinositide response of cultured neonatal rat ventricular myocytes towards ET-1 and PHE. Although major responsiveness of cardiomyocytes to ET-1 and PHE appears to be limited during hypoxia, this agonist did not increase the magnitude of energy depletion caused by 90 min of hypoxia and did not lead to more cell injury (LDH release) during this phase. However, during reoxygenation (15 min) the presence of either ET-1 or PHE was seen to increase cell injury as judged by enzyme leakage and ATP depletion.

Hypoxia/reoxygenation and the phosphoinositide pathway

During glucose-free hypoxia we detected a gradual decline in both ET-1 and PHE induced activation of the phosphoinositide pathway (Figs 1 and 2). In contrast to our results, earlier studies showed that α_1-adrenergic agonist-coupled $InsP_n$ accumulation was increased during early phases of hypoxia due to increased receptor numbers (Heathers *et al.*, 1989; Kagiya *et al.*, 1991; Steinberg and Alter, 1993) and returned to control levels after prolonged hypoxia due to uncoupling of receptors from the effectors (Kagiya *et al.*, 1991). In the studies mentioned above glucose, known to prevent cardiomyocyte ATP levels from becoming depleted during hypoxia (Vemuri *et al.*, 1989), was included in the incubation buffer. Furthermore, in a study of the β-adrenergic receptor-adenylate cyclase system the addition of glucose was shown to change hypoxia-induced inhibition of agonist-dependent cAMP production to hypoxia-induced stimulation, independent of the decrease in β-adrenergic receptors that is brought about by hypoxia (Rocha-Singh *et al.*, 1991). Although we showed that glucose had no effect on hypoxia-induced decrease of ET-1 evoked PLC activity, it is possible that the absence of glucose affects the influence of hypoxia on PHE-induced PLC activity. Recently, Muntz *et al.* showed that 60 min of glucose-free hypoxia led to a small decrease in PHE-induced PLC activity, suggesting that the presence of glucose determines the effect of hypoxia on α_1-adrenergic evoked PLC activity. The fact that ET-1 as well as PHE coupled $InsP_n$ accumulation was diminished by prolonged glucose-free hypoxia suggests that common factors such as PLC or PtdIns(4,5)P_2 are affected. We could indeed show that PtdIns(4,5)P_2 levels were significantly decreased by hypoxia (Fig. 3). However, this decline in PtdIns(4,5)P_2 levels (41% during 90 min of hypoxia) was not as large as the decrease in agonist-induced $InsP_n$ accumulation (62% for ET-1 and 69% for PHE). This suggests that either PLC has only access to a limited pool of PtdIns(4,5)P_2 or that the decrease in PLC substrate levels is not the only cause of attenuated signal transduction. The decline of PtdIns(4,5)P_2 levels during hypoxia was not caused by increased PLC activity itself as no hypoxia-induced $InsP_n$ accumulation was detected. This indicates that increased dephosphorylation of PtdIns(4,5)P_2 or activation of phospholipases A and/or D (PLA and PLD respectively) might have occurred during hypoxia. As no hypoxia-induced rise in PtdIns(4)P (not shown) or PtdIns (Table 1) was detected, PLA or PLD activation seems to be the most logical explanation for reduced PtdIns(4,5)P_2 levels. No rise in lyso-PtdIns(4,5)P_2 was detected after hypoxia (not shown), indicating that complete deacylation or conversion of PtdIns(4,5)P_2 to phosphatidic acid might have occurred. Phospholipid degradation during hypoxia was described before. In one study (Nachas and Pinson, 1992), activation of PLC was suggested to occur during anoxia, leading to a decrease in PtdIns(4,5) but also in PtdIns(4)P and PtdIns level. Release of arachidonic acid from membrane phospholipids is another event accompanying ATP depletion (Chien *et al.*, 1985; Iwaka *et al.*, 1993) implying involvement of phospholipase A_2, although we recently showed that PtdIns(4,5)P_2 is a relatively modest source of arachidonic acid in rat (Lamers *et al.*, 1993a). A third phospholipase demonstrated to be involved in phospholipid degradation during hypoxia/reoxygenation is PLD but it mainly hydrolyzes phosphatidylcholine (Moraru *et al.*, 1992; Lamers *et al.*, 1993b). Taken together,

it is plausible to assume that phospholipases other than PLC were activated during hypoxia in our study, but direct evidence has not yet been obtained.

While hypoxia either decreases (β-adrenergic) or increases (α_1-adrenergic) receptor numbers, reoxygenation is known to return α_1- and β-adrenergic receptor levels to control values (Chien *et al.*, 1985; Heathers *et al.*, 1989; Kagiya *et al.*, 1991). In contrast, ET-1 receptors are increased by hypoxia and even more so during reoxygenation (Liu *et al.*, 1990). In the present study, glucose-free hypoxia-induced inhibition of ET-1 coupled $InsP_n$ accumulation was reversed by reoxygenation, achieved either by normoxic buffer change or by replacement of 95% N_2/5% CO_2 by 95% air/5% CO_2. As ATP levels were still decreased after 15 min reoxygenation, there seems to be no direct correlation between the energy status of the cell and responsiveness to ET-1. This was further confirmed by results from 90 min hypoxia in glucose-containing buffer where ATP levels were only slightly decreased but ET-1 evoked PLC activity was still very low.

The influence of ET-1 on hypoxia/reoxygenation-induced ATP depletion

To establish a cell culture model in which we could study the influence of ET-1 on cell injury during hypoxia/reoxygenation, we had to omit glucose from the incubation buffer, based upon results of previous work by Vemuri *et al.*, 1989 and Rocha-Singh *et al.*, 1991 and as confirmed in this study where glucose prevented ATP depletion and hypoxia-induced cell injury. During glucose-free hypoxia, ATP levels were indeed seen to decrease steadily under these conditions (Fig. 4). Levels of ADP and AMP were also declining during hypoxia (data not shown) indicating that further breakdown of adenine nucleotides occurred, ultimately leading to the formation of adenosine and hypoxanthine which are released into the extracellular environment (Vemuri *et al.*, 1989). Upon 15 min of reoxygenation, a slight but not significant regeneration of ATP was detected. This lack of ATP regeneration might be partially due to the lack of hypoxanthine and glucose in the fresh reoxygenation buffer, providing substrates for the salvage pathway (Harmsen *et al.*, 1984) as reoxygenation without buffer change led to about 50% recover from energy depletion after (prolonged) reoxygenation. ATP levels during 90 min of hypoxia were not changed by the presence of ET-1. Upon reoxygenation the presence of ET-1 led to significantly lower ATP levels. The possible cause for this difference in ATP levels might be the additional membrane leakiness induced by the presence of ET-1 as follows from the LDH release measurements (see below).

ET-1 and cell injury during hypoxia/reoxygenation

During normoxia/buffer change as well as during hypoxia low levels of LDH leaked into the buffer after applying new buffer, independent of the presence of ET-1 (Fig. 6). As no further increase in LDH levels in the extracellular environment was seen during prolonged (30–90 min) incubation, the buffer change-induced shock, probably mechanical in nature, was held responsible for this enzyme leakage. The result that during 90 min of glucose-free hypoxia no LDH leakage was induced corresponds to the observation (Fantini *et al.*, 1990; Iwaka *et al.*, 1993) that only after 120 min of hypoxia significant amounts of LDH or creatine kinase are released in this model, reaching a maximum after 180–210 min. Also as reported before (Fantini *et al.*, 1990), reoxygenation led to increased enzyme leakage, probably reflecting myocardial necrosis. In all experiments performed, the continuous presence of ET-1 led to a (1.7 ± 0.2-fold) increase in LDH release after reoxygenation. Agonist-mediated increase in hypoxia/reoxygenation-induced cell injury was also observed using PHE, showing it not to be receptor-specific. It was reported before (Stawski *et al.*, 1991) that the presence of ET-1 during simulated ischaemia (glucose-free hypoxia together with buffer volume restriction), already led to PKC and Ca^{2+}-dependent increases in LDH release during the hypoxic phase. This ischemic model was furthermore shown to result in disorganized sarcolemmal structures (Ne'eman and Pinson, 1990). The early development of cell damage during simulated ischaemia (Ne'eman and Pinson, 1990) as compared to hypoxia (this study) suggests that either increased extracellular metabolite concentrations can augment ET-1 induced necrosis or that ET-1 induced elevation of extracellular metabolites can aggravate cellular damage.

In conclusion, we showed that glucose-free hypoxia decreases the activity of receptor-mediated phosphoinositide turnover in cultured rat neonatal ventricular myocytes, but even this reduced turnover rate gives rise to enhanced ATP depletion and myocardial necrosis during reoxygenation. The underlying processes leading to increased cell damage are unknown, but might well be ascribed to

e.g. ET-1 induced intracellular Ca^{2+} mobilization (Vigne *et al.*, 1990) and activation of protein kinase C resulting in enhanced Ca^{2+} overload. Whether activation by ET-1 and α_1-adrenergic agonist of Ca^{2+}-dependent proteases (Iizuka *et al.*, 1992) and increased degradation of phospholipids (Jones *et al.*, 1989) contributes to development of myocyte injury remains to be elucidated. As ET and α_1-adrenergic hormones are known to be released during hypoxia and myocardial infarction these agonists might be factors determining the magnitude of cellular damage.

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