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## Research Article

# Pharmacological attenuation of apoptosis in reoxygenated endothelial cells

**A. E. Kabakov[a,*], K. R. Budagova[a], Y. V. Malyutina[a], D. S. Latchman[b] and P. Csermely[c]**

[a] Medical Radiology Research Center, 4 Korolev Street, Obninsk 249036 (Russia), Fax: +7 095 9561440, e-mail: aekabakov@hotmail.com
[b] Institute of Child Health, University College London, London WC1N 1EH (United Kingdom)
[c] Department of Medical Chemistry, Semmelweis University, P. O. Box. 260, 1444 Budapest (Hungary)

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**Abstract.** BRX-235 (Iroxanadine), a novel drug developed by Biorex (Hungary), was previously characterized as a vasculoprotector against atherosclerosis, an activator of p38 kinase, and an enhancer of stress-responsive heat shock protein (Hsp) expression. The present data demonstrate that BRX-235 may improve survival of vascular endothelial cells (ECs) following ischemia/reperfusion stress. ECs cultured from human umbilical veins were exposed to hypoxia/reoxygenation to mimic ischemia/reperfusion. Caspase activation and apoptosis were monitored in the reoxygenated cells. Addition of BRX-235 (0.1–1 μM) to culture medium prior to hypoxia or at start of reoxygenation significantly reduced the caspase-dependent apoptosis. The cytoprotection conferred by the pre-hypoxic drug administration was sensitive to quercetin and seems to be based on enhanced Hsp accumulation in stressed ECs. In the case of post-hypoxic drug administration, the cytoprotection was strongly inhibited by SB202190 and SB203580 and appears to be associated with enhanced p38 kinase activation in reoxygenated ECs.

**Key words.** Apoptosis; heat shock protein; ischemia; reperfusion; MAP kinase.

Vascular endothelial cells (ECs) may become damaged and die in the course of ischemia/reperfusion [1–5], leading to failure of endothelial barrier function, and formation of thrombi and tissue edema. Such vascular disorders, when they arise, aggravate the consequences of an ischemic insult and complicate the outcome of some surgical operations performed with local interruption of blood circulation. Consequently, protection of the vascular endothelium from damage and cell death induced by ischemia/reperfusion is one of the most important tasks in order to avoid post-insult and post-surgical complications. Targeting of special (cytoprotective) drugs to vascular ECs prior to ischemia/reperfusion can yield a therapeutic effect against the impending insult. However, ischemic attacks may develop suddenly and, in such cases, delivery of beneficial drugs will be complicated by blockage of the blood supply to ischemia-affected tissue regions. When blood circulation is restored at the stage of post-ischemic reperfusion, the stressed ECs become accessible to therapeutic agents in the renewed blood flow but, on the other hand, reperfusion induces oxidative stress and apoptosis in the vascular endothelium [3–5]. We need, therefore, to search for pharmacological drugs which are cytoprotective not only during ‘early’ (pre-ischemic) administration but also if they are introduced into the bloodstream at the stage of post-ischemic reperfusion (‘late’ administration).

Heat shock proteins (Hsps) comprise one of the most ancient cellular defense systems conferring cytoprotection against severe ischemic/reperfusion insults [see for

* Corresponding author.

reviews refs 6–8]. Up-regulation of intracellular Hsp levels [9, 10] or stimulation of the p38 stress-activated protein kinase (p38 SAPK)/Hsp27 pathway [11] can protect ECs in experiments with simulated ischemia. This means that non-toxic inducers or enhancers of Hsp expression and/or activators of p38 SAPK might be used as pharmacological remedies for real protection of vascular ECs upon acute ischemia/reperfusion.

BRX-235 (also referred to as Iroxanadine) is a novel non-toxic hydroxylamine-based compound that is a derivative of Bimoclomol [12, 13]. BRX-235 [5,6-dihydro-5-(1-piperidinyl) methyl-3-(3-pyridil)-4H-1,2,4-oxadiazine] was developed in the laboratories of Biorex R&D (Hungary) for treatment of atherosclerosis and its consequences, in particular restenosis and peripheral arterial disease. Among revealed biological activities of BRX-235 is an ability to stimulate migration of vascular ECs via activation (phosphorylation) of p38 SAPK without affecting the level of its expression [14]. Moreover, BRX-235, like Bimoclomol, can enhance the induction and accumulation of Hsps in stressed cells [15]; this effect appears to be realized via the compound-mediated facilitation and prolongation of heat shock transcription factor 1 (HSF1)-dependent transcription of *Hsp* genes [12, 16]. Such properties of BRX-235 suggest that this drug could be able to protect vascular ECs under ischemia/reperfusion.

To examine the supposedly cytoprotective potential of BRX-235, we used an in vitro model of ischemia/reperfusion in which cultured human ECs are exposed to sustained hypoxia followed by reoxygenation; such a stressful treatment leads to caspase-9/caspase-3-dependent apoptosis in reoxygenated ECs [10] and thus to some extent mimics what happens to the vascular endothelium following acute ischemia/reperfusion [3–5]. To model 'early' drug administration (i.e., before an ischemic insult) and 'late' drug administration (i.e., at the stage of reperfusion when the blood circulation is renewed), BRX-235 was added to ECs either prior to hypoxia or at the start of reoxygenation, respectively. As a positive control for the HSF1-dependent up-regulation of Hsp levels in ECs, we overexpressed a constitutively active form of human HSF1 that was previously shown to protect neuronal cells under simulated ischemia [17]. To prevent induction of Hsps in ECs we used quercetin, a well-known blocker of HSF1-dependent gene transcription [18, 19]. p38 SAPK activation in the treated cells was inhibited by SB203580 [14] or by the related inhibitor SB202190 [20].

In the present work, we demonstrate that BRX-235 used at a clinically relevant concentration (0.1 μM) can significantly reduce apoptosis and improve long-term survival in ECs stressed by hypoxia/reoxygenation. The cytoprotective action of BRX-235 is manifested not only when the compound was added to ECs prior to hypoxia ('early' administration) but also in post-hypoxic administration, namely at the start of reoxygenation ('late' administration). These two modes of protection of reoxygenated ECs by BRX-235 seem to be based on two different mechanisms conferring (i) up-regulation of intracellular Hsp levels or (ii) enhanced p38 SAPK activation, respectively. Thus, BRX-235 is characterized here as a promising drug which may attenuate ischemia/reperfusion injury.

## Materials and Methods

### Cells

ECs were isolated from human umbilical cord veins and then cultured on gelatin-coated substrates at 37 °C in a $CO_2$ incubator with a humidified air atmosphere containing 5% $CO_2$ [21]. The culture growth medium consisted of RPMI 1640 supplemented with 10% fetal bovine serum (both HyClone), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml heparin, 20 mM HEPES, and 50 U/ml penicillin (all from Flow Labs). The isolated and grown cell populations were typed as homogenous ECs owing to their total staining with an antibody to factor VIII-related antigen. EC cultures of passages 1–3 were used for all the experiments.

### Simulated ischemia/reperfusion

The EC cultures were incubated at 37 °C in the hypoxic chamber with a humidified atmosphere of 5% $CO_2$ and 95% $N_2$. The residual $O_2$ concentration was monitored using a special $O_2$ electrode (Kendro Laboratory Products) and did not exceed 1%. The cells were exposed to such hypoxia for 20 h and then placed into a $CO_2$ incubator under normal conditions of EC cultivation. Immediately after the hypoxic treatment, the culture medium over the treated cells was replaced with normal growth medium previously conditioned in the $CO_2$ incubator in a normoxic (21% $O_2$) environment. Such a treatment induced sharp reoxygenation, thereby mimicking acute post-ischemic reperfusion in blood vessels [10].

### Drug treatments of ECs

BRX-235 was introduced at different (0.1–1.0 μM) concentrations into the culture medium prior to the start of hypoxia (early administration) or at the start of reoxygenation (late administration). In some EC samples, quercetin (30 μM), SB202190 (1 μM), or SB203580 (1 μM) were added simultaneously with BRX-235. Stock solutions of each drug were prepared on the day of the experiment.

### Overexpression of the constitutively active HSF1 and green fluorescent protein

Construction and generation of herpes simplex virus-based vectors expressing the constitutively active (mutant) form of human HSF1 or the green fluorescent protein (GFP) were described earlier [17, 22]. For effectual infec-

tion, ECs (80% confluence) grown in 6- or 24-well plates were placed into serum-free medium and incubated in contact with the HSF1- or GFP-expressing viral vector (15 p.f.u./ml) for 1 h at 37 °C in the $CO_2$ incubator. After this incubation, the virus-containing medium was replaced with normal (serum-containing) growth medium and the infected ECs remained under normal conditions for 10–12 h until the start of hypoxic treatment.

**Determination of apoptosis and necrosis**

To quantify stress-induced apoptosis, ECs were grown onto round coverslips in 24-well culture plates (Nunc). 3′ ends of DNA in the apoptotic nuclei were labeled with fluorescein-conjugated dUTP using terminal deoxynucleotidyl transferase (Boehringer Mannheim) according to the manufacturer's protocol for the TUNEL assay. The assays were performed after the cells had been fixed with 4% paraformaldehyde for 3 min at 25 °C and washed with PBS three times. The reaction solution containing 2 mM fluorescein-conjugated dUTP and 10 units of terminal deoxynucleotidyl transferase was added to the fixed cells for 2 h in a humidified incubator at 37 °C. After washing with PBS, the cell preparations were analyzed on a fluorescence microscope to calculate the fluorescein-labeled (apoptotic) nuclei as a percentage of total cell nuclei.

The fractions of ECs dying from necrosis were determined after counting the non-fixed cells which became stained with propidium iodide (PI, 10 μg/ml) [10].

**MTT and clonogenic assays**

Post-stress EC viability was assessed in the MTT assay at 14 and 24 h after the start of post-hypoxic reoxygenation [10]. The cells were incubated in the presence of 0.5 mg/ml MTT at 37 °C for 3 h. Then the MTT-containing medium was removed, the cells were washed with PBS and the in situ-generated formazan product was solubilized by adding dimethyl sulfoxide. Absorbance at 630 nm (background) was subtracted from absorbance at 570 nm for each well on a plate reader (Hewlett Packard) [10].

Long-term post-stress survival of the treated cells was evaluated in a clonogenic (colony formation) assay as previously described [23] with minor modifications for ECs. The cells were trypsinized, harvested, and counted, and then the cell suspensions were serially diluted and seeded, in triplicate, in 10-$cm^2$ culture dishes. After 15–17 days growth, the ECs colonies were fixed with 70% ethanol and stained with 0.5% crystal violet. The numbers of separate growing colonies (colonies with a minimum of 50 cells) were counted. The relative clonogenic potential of treated ECs was determined as the ratio of growing colonies formed by the initially plated cells and normalized to the plating efficiency [23]. The average value obtained for unstressed ECs was considered as 100%.

**SDS Electrophoresis and Western blotting**

The cells were washed with PBS and lysed into a reducing Laemmli sample buffer supplemented with inhibitors of proteases [10, 21]. Proteins of the lysate aliquots were run in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrotransferred from gel slabs onto nitrocellulose membrane (Hybond C). After routine blocking step, blots were treated with specific antibodies to human HSF1 (Calbiochem) or to individual Hsps. Hsp70 was revealed with a monoclonal antibody N27F3-4 (StressGen) reacting with both the inducible (Hsp70) and constitutive form (Hsc70). A monoclonal antibody LK-1 (StressGen) was used to detect Hsp60. Hsp27 was detected with rabbit antibodies recognizing both rodent and human Hsp27 (kindly provided by Prof. M. Gaestel).

To assess caspase activation, EC lysates were prepared according to the manufacturer's instructions and run on SDS-PAGE. Immunoblotting was performed with rabbit antibodies to caspase-9 and caspase-3 (Santa Cruz Biotechnology) which recognize both the inactive precursors and the activated (cleaved) forms of either caspase. In addition, caspase-3 activation was evaluated on specific cleavage of poly-ADP ribose polymerase (PARP) using rabbit anti-PARP antibodies (Santa Cruz Biotechnology). Immunoblotting with antibodies to actin (Sigma) was used for control of equal protein loading in all the samples analyzed.

p38 SAPK activation in treated ECs was detected by immunoblotting of the cell lysates with a PhosphoPlus p38 kinase antibody kit (New England Biolabs) [14]. The inhibitory effects of SB203580 and SB202190 on p38 SAPK activation were checked by probing the phosphorylation state of Hsp27 in two-dimensional SDS-PAGE/immunoblotting [24].

After treatment of blots with anti-mouse or anti-rabbit IgG peroxidase conjugates, bands of the immunodetected proteins were visualized as tracks on X-ray film by means of enhanced chemiluminescence (ECL-kit from Amersham). The developed tracks were digitized on a scanner Mustek 600 II CD and then quantitatively analyzed using the NIH Image software [10].

**Statistics**

All quantitative data were calculated here as means ± SE of five to seven separate experiments. Significance of cytoprotection conferred by BRX-235 or activated (mutant) HSF1 overexpression were compared with the control for each group of cells using ANOVA (a multi-way analysis of variance or covariance). ANOVA significance was additionally confirmed with the F test.

## Results

### Pre- or post-hypoxic BRX-235 administration can reduce apoptosis in reoxygenated ECs

Addition of BRX-235 to the culture medium at final concentrations of 0.1–1.0 µM exerted marked cytoprotective effects in this model of ischemia/reperfusion for vascular ECs. Here we illustrate actions of 0.1 µM BRX-235, as this concentration can be reached in patients' plasma and tissues after usual oral doses of the drug (unpublished observations) and was previously shown to activate p38 SAPK in bovine ECs [14]. At 0.1 µM, BRX-235 significantly reduced apoptosis, the predominant form of cell death in reoxygenated ECs (figs. 1, 2). This attenuation of stress-induced apoptosis correlated well with data on the impaired caspase-9/caspase-3 activation (fig. 3) and the improved long-term cell survival following the treatments (figs. 4, 5). Two different protocols of BRX-235 administration, namely (i) prior to hypoxia and (ii) at the start of reoxygenation, yielded rather similar effects on

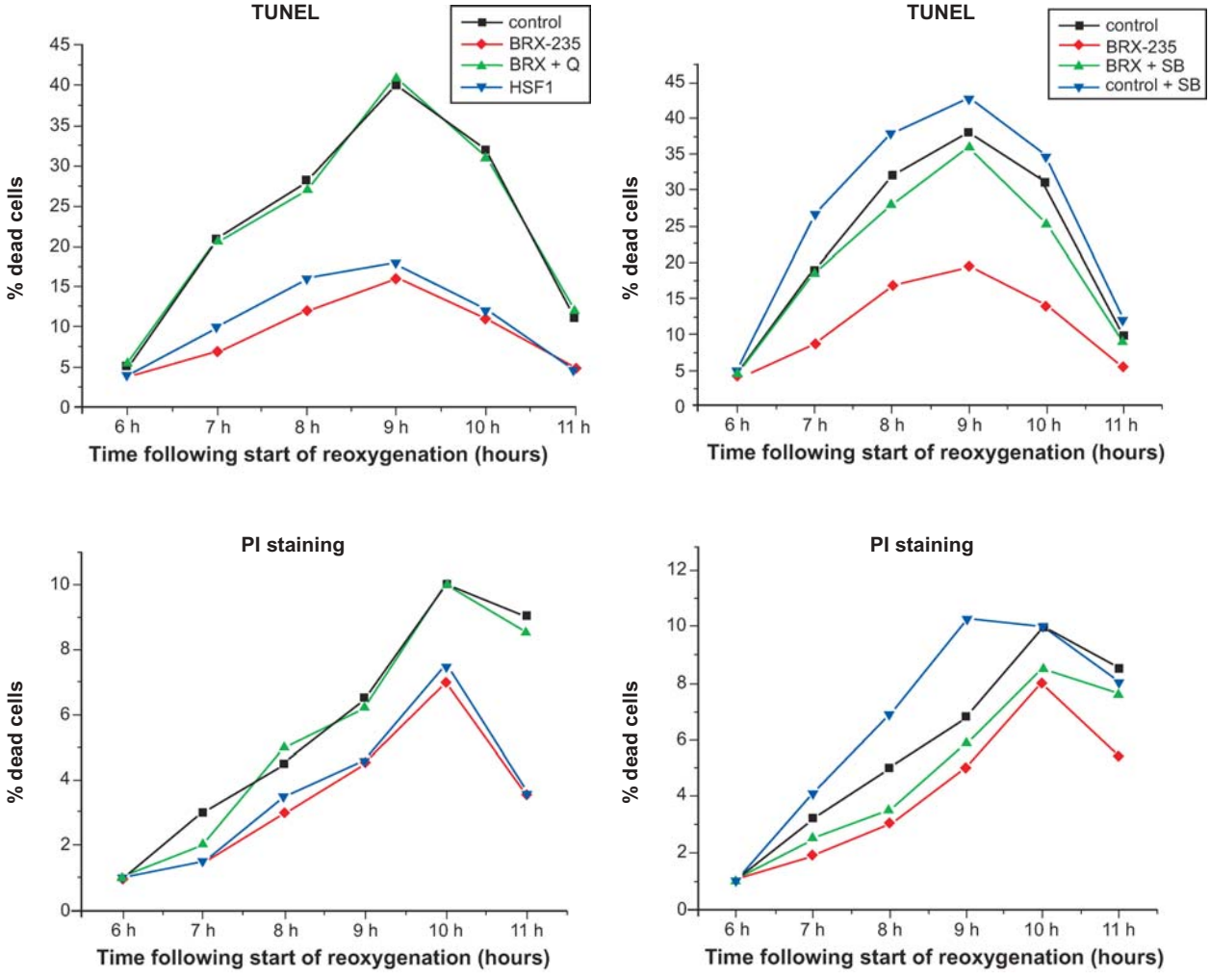


Figure 1. Effects of the pre-hypoxic administration of 0.1 µM BRX-235 with or without 30 µM quercetin (Q) and of the overexpression of activated HSF1 on delayed cell death of ECs subjected to 20 h hypoxia followed by reoxygenation. Overexpression of activated HSF1 was performed as described in Materials and methods. The percentages of dead cells were determined at different time points of reoxygenation after counting apoptotic ECs with TUNEL-positive nuclei, and necrotic (PI-positive) ECs. These data are means of six separate experiments. In the TUNEL assay, a significant difference from the control ($p < 0.05$) was revealed for the BRX-235 and HSF1 groups at 7, 8, 9, 10 and 11 h of reoxygenation. For PI staining, a significant difference from the control ($p < 0.05$) was found for the BRX-235 and HSF1 groups at 11 h reoxygenation.




Figure 2. Effects of post-hypoxic administration of 0.1 µM BRX-235 with or without 1 µM SB202190 (SB) on delayed cell death of ECs subjected to 20 h hypoxia followed by reoxygenation. The percentages of dead cells were determined at different time points of reoxygenation after counting apoptotic ECs with TUNEL-positive nuclei, and necrotic (PI-positive) ECs. All the data presented are means of six separate experiments. In the TUNEL assay, a significant difference from the control ($p < 0.05$) was revealed for the BRX-235 group at 7, 8, 9, 10 and 11 h reoxygenation. No significant difference from the control was found with PI staining.

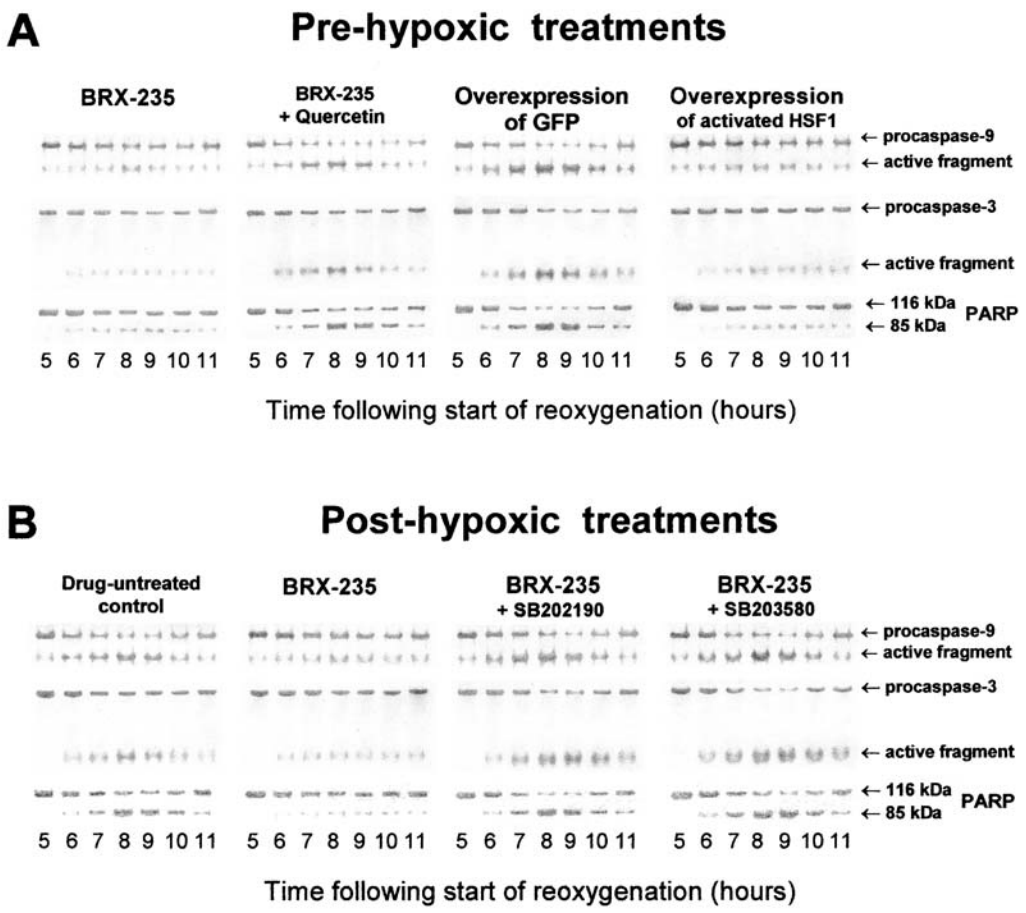


Figure 3. Immunoblots showing the hypoxia/reoxygenation-induced activation of caspase-9 and caspase-3 in ECs treated with 0.1 μM BRX-235 prior to (*A*) or following (*B*) hypoxia and effects of 30 μM quercetin, 1 μM SB202190, 1μM SB203580, and of activated HSF1 or GFP overexpression on caspase activation. All the immunoreactive forms of the caspases and PARP were detected in the EC lysates obtained at different time points of reoxygenation following 20 h hypoxia. The uninfected and drug-untreated control is presented in *B* (left panel). Beside the blots presented, very similar results were obtained in five other independent experiments.

cell death/survival and the caspase activation in reoxygenated ECs (compare data in figs. 1–5). Despite this similarity, we suggest distinct mechanisms for the cytoprotection for pre- or post-hypoxic drug administration (see below).

No improved cytoprotection was observed when BRX-235 was added twice to ECs i.e., prior to and then after hypoxia; in this case, the cytoprotective effects were very close to those exerted by the pre-hypoxic drug administration alone (data not shown).

**Cytoprotective effects of pre-hypoxic BRX-235 administration are abolished by quercetin and mimicked by overexpressed HSF1**

As the revealed cytoprotective properties of BRX-235 could be attributed to its capability to enhance activation of HSF1 and/or p38 SAPK [9–11, 14–16], we probed the effects of quercetin (an inhibitor of HSF1-mediated Hsp expression [18, 19]) and SB203580 and SB202190 (both inhibitors of p38 SAPK [14, 20]). In the case of pre-hypoxic BRX-235 administration, quercetin (figs. 1, 3A, 4A, 5) but not SB202190 (fig. 4A) or SB203580 (not shown) abolished the cytoprotection and the suppression of caspase activation (fig. 3A). Quercetin without hypoxia/reoxygenation affected neither viability of non-stressed ECs (figs. 4A, 5) nor the caspase-9/caspase-3 activity in them (not shown).

The abolishing effects of quercetin implied enhanced expression of cytoprotective Hsp(s) in the drug-treated ECs. To examine this suggestion, we assessed intracellular levels of Hsp70, Hsp60, and Hsp27 (fig. 6, table 1) because these Hsps can protect cells from ischemic stress [9, 10, 25]. It should be noted that the hypoxia/reoxygenation treatment by itself induces the cellular stress response involving HSF1 activation [26, 27] which yielded in our model a slight (1.5- to 1.8-fold) up-regulation of intracellular Hsp levels by 6 h of post-hypoxic reoxygenation (fig. 6, table 1). According to our observations, treatment of ECs with 0.1 μM BRX-235 without hypoxia/reoxygenation did not increase intracellular Hsp levels.

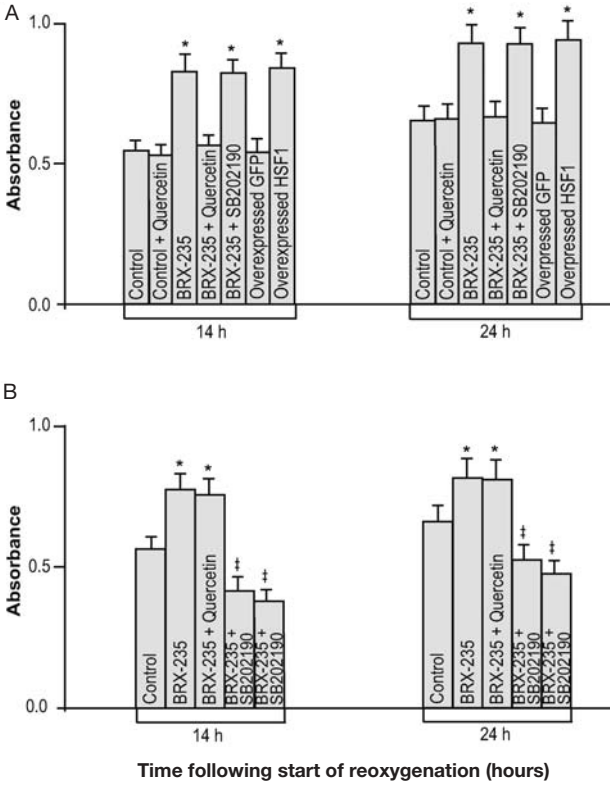


Figure 4. Effects of pre-hypoxic (*A*) and post-hypoxic (*B*) administration of 0.1 μM BRX-235, with or without 30 μM quercetin, 1 μM SB202190, or activated HSF1 or GFP overexpression on EC viability determined in the MTT assay at 14 h and 24 h following the start of post-hypoxic reoxygenation. Data are means ±SE of six separate experiments. Significant differences from the control: $^{*}p < 0.01$, $^{‡}p < 0.05$.

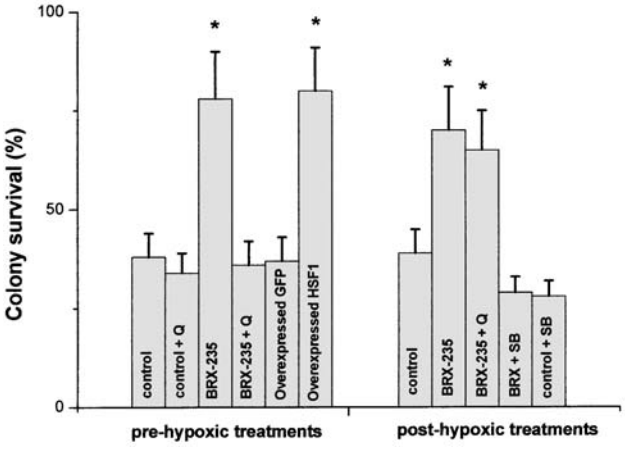


Figure 5. Effects of pre-hypoxic and post-hypoxic administration of 0.1 μM BRX-235, with or without 30 μM quercetin (Q), 1 μM SB202190 (SB), or GFP or HSF1 overexpression on the ability of ECs to form viable cell colonies following 20 h hypoxia. The numbers of surviving colonies for each group were normalized to the unstressed cells: the average value obtained for untreated ECs growing under normal conditions was taken as 100% of possible surviving colonies. Colony survival in all other groups of ECs is expressed as a percentage of that in the unstressed cells. The group where ECs underwent hypoxia without any additional treatments is designated here as control. These data are means ±SE of four separate experiments. Significant difference from the control: $^{*}p < 0.01$.

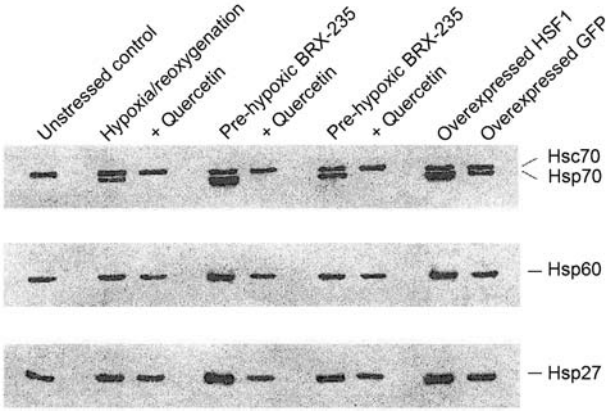


Figure 6. Immunoblots showing bands of Hsc70/Hsp70, Hsp60, and Hsp27 in samples of ECs treated with 0.1 μM BRX-235 prior to or following hypoxia, with or without 30 μM quercetin, and in ECs overexpressing either GFP or the activated HSF1. Beside the blots presented, very similar results were obtained in five other independent experiments. The average intracellular Hsp contents determined after quantitative analyses of these and the other blots are presented in table 1.

However, Hsp accumulation in ECs pre-treated with BRX-235 before hypoxia was significantly (2- to 2.8-fold) greater than in the drug-untreated cells (fig. 6, table 1). Immunofluorescent staining and flow cytometry analyses revealed a rather even distribution of Hsp70, Hsp60, and Hsp27 in populations of these cells (data not shown).

In parallel, we compared the effects of the pre-hypoxic BRX-235 treatment with effects of overexpression of the constitutively active (mutant) form of HSF1. Overexpression of GFP was here used as a control. The intracellular accumulation of either overexpressed product was confirmed by immunoblotting and flow cytometry analysis. The HSF1-overexpressing cells up-regulated levels of Hsp70/Hsc70, Hsp60, and Hsp27 (fig. 6, table 1) and, under post-hypoxic reoxygenation, exhibited impaired caspase activation (fig. 3A), a lower intensity of apoptosis (fig. 1), and improved long-term survival (figs. 4A, 5); all these findings seem very similar to those obtained in ECs treated with BRX-235 prior to hypoxia. Levels of Hsp expression and cell death/survival in GFP-overexpressing ECs were the same as in the uninfected control (figs. 4A, 5, table 1).

## Cytoprotective effects of post-hypoxic BRX-235 administration are abolished by inhibition of p38 SAPK

Post-hypoxic BRX-235 administration only slightly increased intracellular Hsp levels (fig. 6, table 1); the cytoprotection in this case was unaffected by quercetin but was strongly depressed by the inhibitors of p38 SAPK, SB202190 and SB203580 (see figs. 2, 4B, 5). These data

Table 1. Relative Hsp levels in ECs following hypoxia/reoxygenation, pre- or post-hypoxic administration of BRX-235, and overexpression of GFP or the constitutively activated HSF1.

| Sample | Relative Hsp contents in cells (%) | | |
|---|---|---|---|
| | Hsc70/Hsp70[a] | Hsp60 | Hsp27 |
| Control uninfected cells[b] | 183 ± 28 | 162 ± 20 | 165 ± 25 |
| + quercetin[c] | 109 ± 13‡ | 105 ± 14‡ | 103 ± 10‡ |
| Pre-hypoxic BRX-235 administration | 495 ± 58* | 288 ± 28* | 347 ± 35* |
| + quercetin[c] | 111 ± 12‡ | 104 ± 9‡ | 99 ± 8‡ |
| Post-hypoxic BRX-235 administration | 216 ± 31 | 173 ± 26 | 181 ± 29 |
| + quercetin[c] | 113 ± 12‡ | 107 ± 10‡ | 102 ± 11‡ |
| Overexpression of active HSF1 | 461 ± 52* | 282 ± 33* | 323 ± 37* |
| Overexpression of GFP | 185 ± 27 | 147 ± 24 | 153 ± 32 |

Shown are relative Hsp contents in ECs subjected to 20 h hypoxia followed by 6 h reoxygenation. The average content of each Hsp determined for untreated ECs was considered to be 100%. Data are means ± SE of six separate experiments. * Significant difference from unstressed, uninfected control, $p < 0.01$. ‡ Significant difference from stressed/BRX-235-treated samples, $p < 0.05$.

[a] The relative Hsc70/Hsp70 content in the treated ECs was assessed as the integral level of heat shock cognate protein 70 (Hsc70) + inducible Hsp70.

[b] The relative contents of Hsc70/Hsp70, Hsp60, and Hsp27 are increased in this group vs the untreated control (100%); this increase appears to be due to Hsp induction in response to the hypoxia/reoxygenation stress.

[c] In these groups, quercetin (30 μM) was added to ECs prior to the start of hypoxia.

are in accordance with the fact that both SB202190 and SB203580 abolished the suppressive effect of post-hypoxic BRX-235 administration on caspase-9/caspase-3 activation (fig. 3B).

As in vivo p38 SAPK undergoes stress-responsive phosphorylation resulting in activation of the kinase [28, 29], the phosphorylation status (and therefore the extent of activation) of p38 SAPK was analyzed here using anti-phospho-p38 kinase antibodies (fig. 7). Taking into consideration that SB202190 and SB203580 inhibit enzymatic activity of p38 SAPK without affecting its phosphorylation [20], we revealed the action of either inhibitor by monitoring a suppression of phosphorylation of Hsp27 (a downstream substrate in the p38 SAPK cascade [28, 29]) (fig. 8). At 0.1 μM, BRX-235 activated p38 SAPK (fig. 7A) and induced phosphorylation of Hsp27 in unstressed human ECs (fig. 8; top panel), in agreement

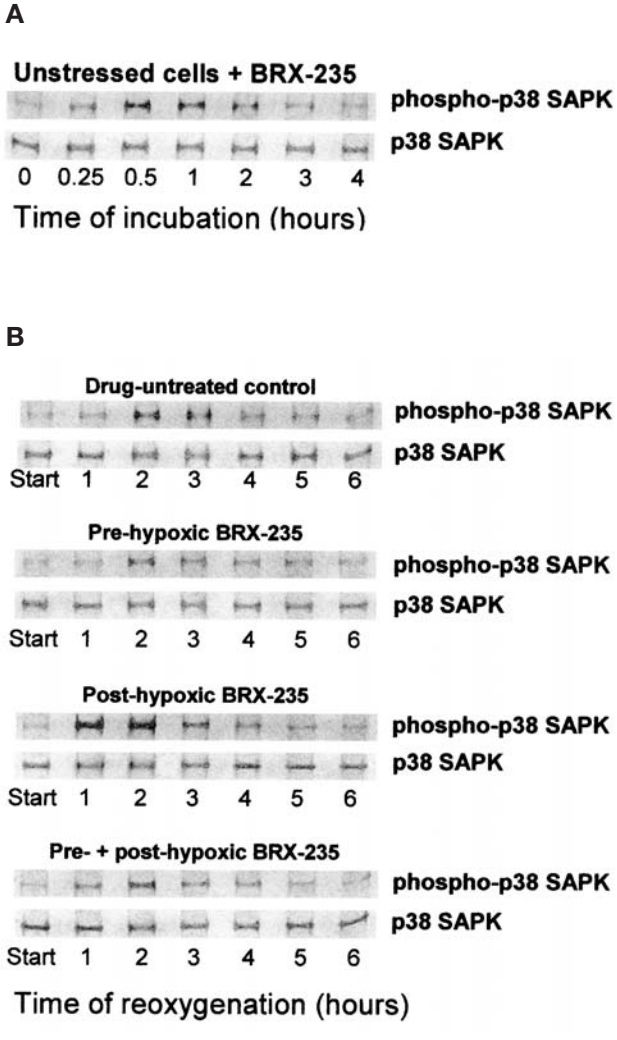


Figure 7. Activation of p38 SAPK in ECs subjected to hypoxia/reoxygenation and/or treatment with BRX-235 prior to or following hypoxia: immunoblots showing the phosphorylation (activation) of p38 SAPK in ECs at different time points of their incubation with 0.1 μM BRX-235 (*A*) and immunoblots showing how 0.1 μM BRX-235 affects the phosphorylation (activation) of p38 SAPK in ECs exposed to hypoxia/reoxygenation (*B*). In each pair of blots, the upper demonstrates development with the anti-phospho-p38 SAPK antibodies, reflecting the level of enzyme activation, while the lower demonstrates the same membrane stripped and reprobed with anti-p38 SAPK, reflecting the protein contents of the enzyme in the same sample of the cell lysates. Unstressed cells (*A*) did not experience hypoxia/reoxygenation, while all the other samples (*B*) were subjected to 20 h hypoxia (Start) followed by 1–6 h of reoxygenation as indicated. Very similar blots were also developed in other four independent experiments.

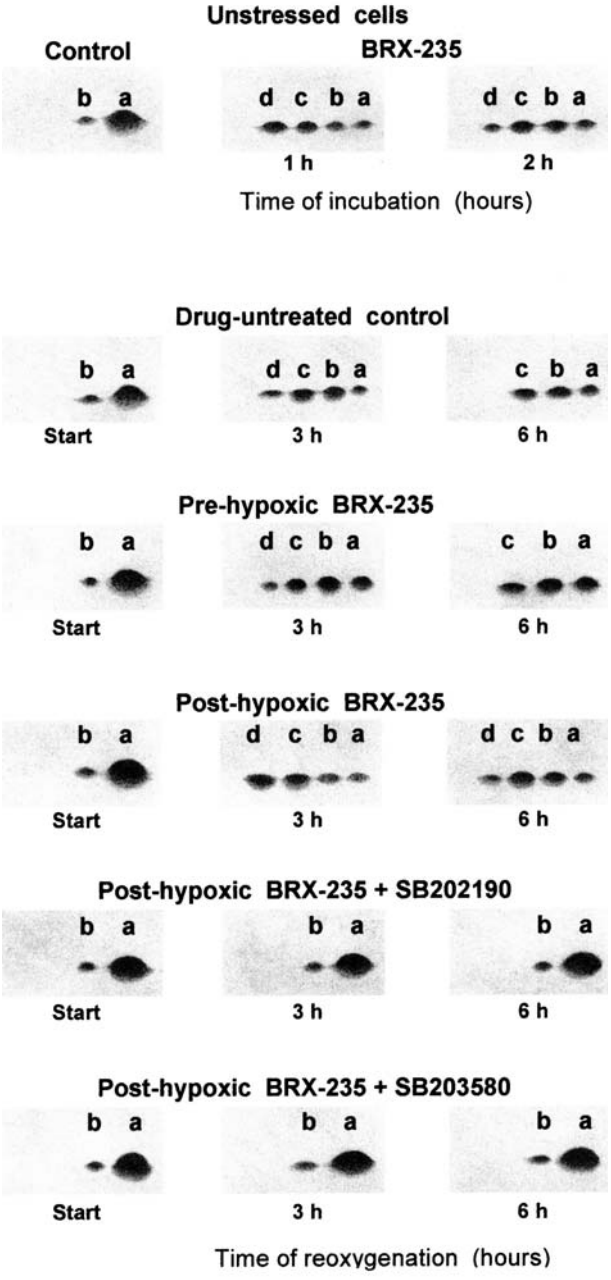


Figure 8. The isoform patterns of Hsp27 revealed by two-dimensional immunoblotting in samples of unstressed ECs and ECs exposed to post-hypoxic reoxygenation in the presence or absence of 0.1 µM BRX-235, 1 µM SB202190 and 1 µM SB203580. Unstressed cells did not experience hypoxia/reoxygenation, whereas all other samples (drug-untreated control and BRX-treated cells) were subjected to 20 h hypoxia (Start) followed by 3 and 6 h of reoxygenation as indicated. These ECL-developed images show the patterns of unphosphorylated (a), monophosphorylated (b), diphosphorylated (c) and triphosphorylated (d) isoforms of human Hsp27. Very similar results were obtained in three other independent experiments.

with previous data obtained for bovine ECs [14]. As for the stressed cells, transient phosphorylation of p38 SAPK (fig. 7B, 'Drug-untreated control') and the apparent increase in phospho-isoforms of Hsp27 (fig. 8, 'Drug-untreated control') indicate that even without the drug treatment, p38 SAPK is activated in reoxygenated ECs. In contrast to the pre-hypoxic treatment of ECs with BRX-235, post-hypoxic treatment markedly enhanced p38 SAPK activation occurring under reoxygenation (see figs. 7B, 8). In fact, post-hypoxic BRX-235 administration yielded an earlier and greater activation of p38 SAPK, as compared with ECs reoxygenated in the absence of the drug (fig. 7B). Meanwhile, the Hsp27 isoform patterns (fig. 8) indicate that both SB202190 and SB203580 did suppress this BRX-235-enhanced p38 SAPK activation. Taking into account that both the inhibitors significantly decreased post-stress EC survival (see figs. 2, 4A, 5; results obtained with SB202190 were identical to those with SB203580) we suggest that (i) the stress-responsive p38 SAPK activation plays a cytoprotective role in our model and (ii) the cytoprotective action of post-hypoxic BRX-235 administration is mainly associated with enhanced p38 SAPK activation in reoxygenated ECs.

Interestingly, p38 SAPK activation during reoxygenation was somewhat impaired in ECs pre-treated with BRX-235 before hypoxia compared with the drug-untreated control (fig. 7B). When BRX-235 was added twice to ECs, prior to and then after hypoxia, p38 SAPK activation during reoxygenation was impaired compared with the effect of post-hypoxic addition of BRX-235 alone (fig. 7B). Such an impairment of p38 SAPK activation appears to explain the absence of improved cytoprotection under the double (pre- + post-hypoxic) treatments of EC with BRX-235.

## Discussion

Our results show that 0.1 µM BRX-235 can attenuate apoptosis in vascular ECs subjected to hypoxia/reoxygenation. The cytoprotection conferred by pre-hypoxic BRX-235 administration seems to be caused by enhanced expression of cytoprotective Hsps. Indeed, a significant accumulation of Hsp70/Hsc70, Hsp60, and Hsp27 in BRX-235-pretreated ECs (see fig. 6, table 1) may be responsible for the improved cell resistance to hypoxia/reoxygenation, as was found earlier in similar models with overexpression of these Hsps [9, 10, 22, 25]. Hsp70, Hsp60, and Hsp27 are major molecular chaperones which may protect cellular proteins from the proteotoxic impact of hypoxia/reoxygenation, thereby attenuating the stress-associated cytotoxicity. Moreover, some researchers consider Hsp70 and Hsp27 as potential suppressors of caspase-dependent apoptosis which are able to block certain stages in the apoptotic pathway thus switching the cell

program from suicide to survival [see for a review refs 30, 31]. In turn, excess Hsp60 may ensure better preservation and/or recovery of mitochondria following hypoxia/reoxygenation and prevent apoptosis in the stressed cells [25]. The data of the experiments with quercetin and overexpression of the constitutively active HSF1 strongly support our suggestion for cytoprotective Hsp(s) whose expression in reoxygenated ECs is enhanced by pre-treatment with BRX-235.

At present, we can only speculate about potential mechanisms of the BRX-235-induced enhancement of endogenous Hsp expression. BRX-235 may promote faster activation and/or nuclear translocation of HSF1 in hypoxic and reoxygenated ECs or, similar to the mechanism of the structurally related compound, Bimoclomol [16], BRX-235 may induce a prolonged binding of activated HSF1 to the respective heat shock element in the *Hsp* gene promoter regions. Other potential mechanisms affecting Hsp mRNA stability, translation machinery and/or Hsp turnover cannot yet be excluded; however, their negligible contribution to the Bimoclomol action [16] makes these possibilities rather unlikely.

We found that two selective inhibitors of p38 SAPK, SB202190 and SB203580, aggravate the delayed cell death occurring upon reoxygenation in both BRX-235-treated and -untreated ECs (figs. 2, 4). This suggests a protective role for p38 SAPK activation in the EC response to hypoxia/reoxygenation. Other researchers [11] have already reported that artificial (achieved by estradiol E2) p38 SAPK activation in cultured bovine ECs protects them from hypoxia-induced apoptosis. Similarly, stimulation of the p38 SAPK/Hsp27 pathway in bovine ECs treated with tumor necrosis factor-alpha was suggested to perform an anti-apoptotic function [32]. Consequently, the BRX-235-enhanced activation of p38 SAPK in reoxygenated ECs seems to be responsible (at least in part) for the elevated cell resistance to apoptosis. Because the BRX-235-induced hyperactivation of p38 SAPK in ECs results in transient accumulation of phosphorylated Hsp27 (fig. 8), the latter may contribute to better preservation/recovery of cellular F-actin and protection from caspase-dependent apoptosis, as was demonstrated earlier in other models [29, 33, 34]. However, both the physiological role of p38 SAPK activation in response to ischemia/reperfusion and the effects of BRX-235 on this activation require, of course, more detailed investigations. On one hand, enhanced activation of p38 SAPK in ischemia/reperfusion-stressed ECs may be beneficial for post-stress cell survival [11, 32, present work]. On the other hand, excessive activation of p38 SAPK in vascular ECs may lead to increased permeability of the endothelial barrier [35, 36] and expression of cell adhesion molecules (ICAM-1, E- and P-selectins) on the cell surface [37, 38] thereby promoting development of edema, thrombosis and tissue-damaging neutrophil attack.

It seems, at first glance, strange that the pre-hypoxic BRX-235 treatment attenuated p38 SAPK activation under subsequent reoxygenation, even in the case of the double (pre- + post-hypoxic) addition of the drug to ECs (fig. 7B). However, excess Hsp70 [39] and Hsp27 [40] in stressed cells have previously been described to suppress the activation of p38 SAPK in response to simulated ischemia/reperfusion. In our model, p38 SAPK activation in the BRX-235-pretreated ECs was also impaired (fig. 7B), presumably through the enhanced intracellular accumulation of Hsp70 and Hsp27 (see fig. 6, table 1). The accumulating Hsps transiently form the stress-resistant cellular phenotype and such Hsp-enriched (tolerant) cells generally exhibit attenuated responses (e.g., impaired activation of p38 and JNK kinases [39–41]) to stressful exposures. In any event, one should take into account that the BRX-235-conferred increase in intracellular Hsp levels may weaken some biological effects based on the stress-responsive p38 SAPK activation.

Nevertheless, the ability of BRX-235 to enhance beneficially endogenous Hsp expression under hypoxia/reoxygenation appears quite hopeful in the context of pharmacological overcoming ischemia/reperfusion injury. A concentration of 0.1 μM BRX-235 can protect human ECs stressed by hypoxia/reoxygenation, and such a concentration may be readily achieved in the clinical setting to achieve the therapeutic effect toward the ischemia/reperfusion stress. Although ischemia/reperfusion by itself activates HSF1 and induces Hsps [42, 43], the in vivo cytoprotective capacity of such an adaptive response is, apparently, insufficient to withstand the acute insult. Thus, artificial gene constructs expressing the constitutively activated (mutant) HSF1 [17, 44] or certain Hsps [6–10, 22, 25] and special (e.g., virus-based) vectors for delivery of such constructs into target cells have been discussed as potential tools for gene therapy of ischemic injury. However, the clinical use of gene therapy against ischemic injury is still in the long term. For the moment, it seems more realistic to develop appropriate pharmacological inducers or enhancers of the expression of cytoprotective Hsps (e.g., Hsp70, Hsp60, Hsp27) in human tissues suffering from ischemia/reperfusion. Our data showing that 0.1 μM BRX-235 can enhance 2- to 2.8-fold the endogenous expression of these Hsps in hypoxia/reoxygenation-stressed ECs may be of clinical significance. Administration of BRX-235 in patients with myocardial or cerebral ischemia may beneficially up-regulate the Hsp levels in their tissues affected by the pathology. Moreover, the BRX-235-conferred enhancement of Hsp expression could be used to attenuate harmful consequences of the reperfusion stress that often occur in the course of surgical operations with bypass perfusion, cardioplegia, or transplantation of organs.

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