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Homologous Adaptation to Oxidative Stress Induced by the Photosensitized Pd-bacteriochlorophyll Derivative (WST11) in Cultured Endothelial Cells*

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Various forms of cellular stress induce adaptive responses through poorly understood mechanisms. In maintaining homeostasis, endothelial cells respond and adapt to changes in oxidative stress that prevail in the circulation. Endothelial cells are also the target of many oxidative stress-based vascular therapies. The objectives of this study were to determine whether endothelial cells adapt to oxidative stress induced upon the photosensitization of WST11 (a water-soluble Pd-bacteriochlorophyll derivative being developed as a photodynamic agent) and to study possible cellular mechanisms involved. The hallmark of WST11-based photodynamic therapy is the *in situ* generation of cytotoxic reactive oxygen species causing vascular shutdown, hypoxia, and tumor eradication. Here we demonstrated that photodynamic therapy also induces adaptive responses and tolerance following a sublethal preconditioning of endothelial cells with the same (homologous) or different (heterologous) stressor. A link among p38 MAPK activity, expression of hsp70 and hsp27, and homologous adaptation to reactive oxygen species induced by photosensitized WST11 was established. In addition to characterization of some key proteins involved, our observations provide a beneficial new working tool for the studies of mechanisms involved in oxidative stress and adaptation using light-controlled photosensitization.

Oxidative stress can trigger two opposing cellular responses depending on the severity of the induced stress, one leading to cell death and the other to transient non-lethal physiological changes. A major feature of the physiological response to oxidative stress is its adaptive and protective nature. Adaptation or tolerance to stress can be defined as the ability of a cell or an organism to become resistant to stress following a sublethal stress experience (1). For instance, clinically relevant adaptation has been mentioned with respect to protection of the heart myocardium and other organs against ischemia and reperfusion injury (2). The adaptation process is time-dependent and requires physiological rearrangement. Evidently, if cells are sensitized by oxidative stress at low levels, tolerance to a second oxidative challenge will probably be manifested within 16–24 h (3).

Oxidative stress is the basis of photodynamic therapy (PDT)[1] where tumors are destroyed by an overwhelming burst of cytotoxic reactive oxygen species (ROS) generated upon local *in situ* photosensitization of an administered photosensitizer (4). *In situ* generation of ROS by photosensitization of preaccumulated pigments in cultured tumor cells has been used for the elucidation of the molecular basis of PDT (5). Endothelial cells (ECs) serve as a major target in anti-vascular PDT induced by bacteriochlorophyll derivatives (6–12). Furthermore, ECs are most sensitive to rapid oxidative changes in the circulation and are presumably capable of adapting to these changes. Consequently, cultured H5V mouse ECs were chosen as a model in this study of adaptation to oxidative stress.

The basis of adaptation to photocytotoxic stress and resistance to PDT therein have not been studied in detail. This is specifically true in cases of homologous adaptation where induction of tolerance, *i.e.* preconditioning and the probing challenge are both induced by photogenerated ROS (pROS), but also in heterologous adaptation (cross-tolerance) where ROS in both phases originates from different processes. Moreover, the mechanisms of adaptation and altered gene expression are largely unknown.

This study focuses on homologous adaptation using photosensitized WST11 (a water-soluble Pd-bacteriochlorophyll derivative) (13), whereas heterologous adaptation, using H_2O_2 for preconditioning, was employed for comparison. Hydrogen peroxide was chosen as a heterologous stressor because of its evolution in cells following ROS formation and because it is a common oxidative stress inducer in the studies of adaptation to oxidative stress (3, 14). It is of importance to study homologous adaptation, which is entirely based on pROS, not only when considering sequential PDT sessions in the clinical setting but also as an advantageous tool for controlled-ROS generation[2] in

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[1] The abbreviations used are: PDT, photodynamic therapy; C cells, challenged cells; CHX, cycloheximide; ECs, endothelial cells; HSP, heat shock protein; LD, lethal dose; MAPK, mitogen-activated protein kinase; MAPKAPK-2, MAPK-activated protein kinase 2; P cells, preconditioned cells; ROS, reactive oxygen species; pROS, photogenerated ROS; SB, SB202190; WST11, a water-soluble Pd-bacteriochlorophyll derivative; ERK, extracellular signal-related kinase; JNK, c-Jun NH_2-terminal kinase.

[2] Y. Posen, R. Seger, A. Brandis, A. Scherz, and Y. Salomon, manuscript in preparation.

Time after start of adaptation experiment (h): -24 Cell Platting; 0 Preconditioning; 24 Challenge; 48 Survival Assay

SCHEME 1. **Adaptation protocol time course.**

the studies of adaptive processes, which are exemplified here.

Adaptation to oxidative stress induced by various stressors involves changes in expression and/or activity of numerous cellular proteins (15, 16). There is strong evidence that the induction of heat shock proteins (HSPs) coincides with the acquisition of tolerance to stress, which could otherwise lead to cell kill (17). HSPs have strong cytoprotective effects, they are involved in various regulatory pathways, and behave as molecular chaperones for cellular proteins by assisting in their correct folding (1, 18). HSPs are important modifying factors in cellular responses to a variety of physiologically relevant conditions such as hyperthermia, exercise, metabolic challenge, aging, and oxidative stress (1, 16, 19). Furthermore, lipid peroxides that are also a feature of bacteriochlorophyll-based PDT (8, 10) were shown to directly participate in the induction of cytoprotective, stress-tolerance-induced proteins such as HSPs, as demonstrated in a mouse brain model (20). Antioxidants such as the ROS scavengers CuZn superoxide dismutase 1 and catalase may also undergo changes (level and activity) in response to oxidative stress (3, 21).

It is becoming evident that ROS plays a central role in cellular signaling by direct alterations of protein kinases and phosphatases (22, 23). Earlier results of our laboratory[2] have demonstrated that among several mitogen-activated kinases (MAPKs), p38 MAPK was activated upon photosensitization with sublethal doses of Pd-bacteriochlorophyll-serine and proposed to have a central role in mediation of pROS-induced oxidative stress. Photosensitization using other sensitizers was also shown to induce p38 activation (24, 25). Because p38 is linked to the regulation of genes involved in various cellular responses, preconditioning, and adaptation (26–29) to various stressors, it is likely to play a role in the regulation of homologous adaptation to pROS. In this study, we demonstrated the induction of an adaptive response and tolerance to pROS in ECs, following preconditioning with sublethal doses of homologous or heterologous oxidative stressors. We examined the induction of various HSPs and antioxidants and their possible role in the development of homologous adaptation. The link between homologous adaptation to pROS and *de novo* expression of hsp70 and hsp27, as well as a possible involvement of p38 in the regulation of this process, were determined in an attempt to elucidate the complex mechanism of this phenomenon.

EXPERIMENTAL PROCEDURES

Materials

Rabbit anti-inducible hsp70 antibodies were purchased from Stressgen Biotechnologies Corporation (Victoria, British Columbia, Canada). Goat anti-hsp27 antibodies and rabbit anti-CuZn superoxide dismutase 1 were purchased from Santa Cruz Biotechnology, Inc. Rat anti-hsp90 antibodies (Stressgen) were kindly provided by Professor Y. Yarden, of our department. Rabbit anti-hsp60 antibodies were kindly provided by Professor Y. Cohen, The Weizmann Institute, Rehovot, Israel. Goat anti-catalase antibodies were purchased from the Binding Site, Ltd. (Birmingham, UK). Mouse anti-phosphorylated-p38 and rabbit anti-general p38 antibodies (Sigma) were kindly provided by Professor R. Seger, of our department. Rabbit anti-phosphorylated-MAPKAPK-2 (Thr-334) antibodies were purchased from Cell Signaling Technology. Horseradish peroxidase-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. Alkaline phosphatase-conjugated secondary antibodies, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl-phosphate were purchased from Promega. Mouse anti-β-actin antibodies, cycloheximide, phenylmethylsulfonyl fluoride, leupeptin, benzamidine, aprotinin, and pepstatin were purchased from Sigma. SB202190 (SB) was purchased from Calbiochem-Novabiochem Corporation. Western blotting luminal reagent was purchased from Santa Cruz Biotechnology, Inc. Neutral red was purchased from Fluka (Buchs, Switzerland). All other reagents were of an analytical grade.

Methods

Cell Culture

Heart mouse embryonic endothelial cells (H5V) monolayers (30) were cultured (up to ~50 passages) in Dulbecco's modified Eagle's medium/F12 containing 15 mM HEPES, pH 7.4, 1.2 mg/l sodium bicarbonate, 10% fetal calf serum, 2 mM glutamine, 0.06 mg/ml penicillin, and 0.1 mg/ml streptomycin (Biological Industries, Bet Haemek, Israel). Cells were grown at 37 °C in an 8% CO_2-humidified atmosphere.

Pigment Synthesis

Palladium 3^1-oxo-15-methoxycarbonylmethyl-rodobacteriochlorin 13^1-(2-sulfo-ethyl) amide dipotassium salt (WST11) was prepared as described earlier (13). Pigments were dissolved in cell culture medium.

Light Source and Illumination

A light field provided by 4 × 100 W halogen lamps (Osram, Germany) equipped with a high pass cut off filter ($\lambda > 650$ nm) and a 4-cm water filter was used as a light source. Illumination was delivered from the bottom of the culture plate at a dose of 12 J/cm^2.

Photosensitization of Cells Pretreated with WST11 (Induction of pROS)

Cells were seeded (12.5×10^4 cells/well) in 12-well plates and cultured for 24 h. The culture medium was replaced, and the cells were preincubated (1 h, 37 °C) with the indicated concentrations of WST11. The culture medium was replaced with fresh medium to remove free sensitizer immediately before cells were illuminated for 10 min at room temperature. The cells were then placed in the culture incubator (24 h, 37 °C). Cell survival was determined by neutral red accumulation according to Ref. 31. After the subtraction of assay blanks, the net optical density (570 nm) was computed as the mean of triplicate determinations ± S.E. Lethal doses (LDs) were determined by calculating the extent of dead cells upon photosensitization as a percentage of the control.

Adaptation Protocol

The protocol involves two sequential steps in which cells were exposed to oxidative stress, (i) preconditioning at sublethal doses of oxidative stress (by either pROS or H_2O_2) and (ii) challenge with lethal doses of pROS.

Preconditioning with pROS—Naive cells were subjected to photosensitization with the indicated WST11 concentrations ($\leq LD_{50}$) and placed in the culture incubator (24 h, 37 °C).

Preconditioning with H_2O_2—Naive cells were preincubated with the indicated concentrations of H_2O_2 and placed in the culture incubator (24 h, 37 °C).

Challenge with pROS—Naive or preconditioned cells (P cells) were photosensitized with the indicated WST11 concentrations ($> LD_{50}$). Cell survival was determined at 24 h after challenge (48 h after the start of the preconditioning step). The time course of the adaptation protocol is summarized in Scheme 1. In all adaptation experiments, cell survival was calculated as the percent of the neutral red accumulated in the corresponding P cells.

Controls—The adaptation experiments included the controls described in Table I. WST11 at all concentrations used exhibited no dark toxicity (13).

Inhibition of Adaptation

Preconditioning with pROS of SB202190-treated Cells—Cells were preincubated with WST11 for 30 min upon which Me_2SO was added to a final concentration of 0.1% with or without SB (final concentration 10 μM) until the end of the preincubation time (1 h). The cells were then washed with fresh culture medium containing Me_2SO with or without SB, respectively (at the concentrations above), and immediately illumi-

TABLE I
Experimental and control groups used in adaptation experiments

Group	Preconditioning[b]		Challenge	
	WST11	Light	WST11	Light
Preconditioning + challenge	+	+	+	+
Preconditioning control	+	+	−	+
Challenge control	−	+	+	+
Light control	−	+	−	+
Untreated control[a]	−	−	−	−

[a] Additional control only for Western blot analyses.
[b] When using H_2O_2 for preconditioning, control cells for this step were kept in the incubator for additional 24 h until the challenge.

nated. The culture medium of all cells was replaced with fresh medium at 8 h postillumination.

Preconditioning with pROS of Cycloheximide-treated Cells—Cells were preincubated with WST11 for 1 h and just before illumination, ethanol was added to a final concentration of 0.1% with or without cycloheximide (CHX) (final concentration 0.1 mM). The culture medium of all cells was replaced with fresh medium at 6 h postillumination.

Preparation and Analysis of Cell Lysates

Cell extracts were prepared in a radioimmune precipitation assay lysis buffer (32), and proteins (30–60 μg of protein/lane) were subjected to a 10–12.5% SDS-PAGE, blotted onto nitrocellulose membranes and immunodetected using the indicated antibodies. All quantifications of the Western blots were performed by reprobing the blots with an antibody to β-actin followed by densitometry. After scanning, band densities were determined (Quantity One, Bio-Rad).

Statistical Analysis

All cell survival experiments were conducted in triplicate determinations and presented as the mean ± S.E. All *t* tests were two-tailed with p values < 0.05. All of the experiments described in this study were conducted at least three times, and representative experiments are shown.

RESULTS

Homologous Adaptation, Resistance of H5V Cells to pROS Challenge following Preconditioning with pROS—The major ROS generated by photosensitized WST11 are singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot-}$), and hydroxyl radicals ($OH^{\cdot}$) as determined by electron spin resonance spectroscopy (33), with a probable endogenous secondary evolution of hydrogen peroxide (H_2O_2) (5). The phototoxicity of photosensitized WST11 ($LD_{50} \approx 3$ μM) in cultured H5V monolayers has been established previously in our laboratory (13) and was shown to be sensitized and light-dependent. As described in Fig. 1, the preconditioning (P) of cells for oxidative stress was performed at 1, 2, or 3 μM ($P_{(1),\ (2),\ \text{or}\ (3)}$, corresponding to LD_5, LD_{20}, or LD_{50}, respectively), whereas the challenge (C) was performed at 5 or 10 μM ($C_{(5)\ \text{or}\ (10)}$, corresponding to LD_{80} or LD_{90}, respectively). As can be seen from this experiment, the survival of non-adapted ($P_{(0)}$) $C_{(5)}$ cells was only 21%, whereas that of adapted $P_{(1),\ (2)}$ + $C_{(5)}$ cells increased to 28 and 55%, respectively (Fig. 1*A*). The survival of non-adapted ($P_{(0)}$) $C_{(10)}$ cells was 8% and increased to 46% in adapted $P_{(3)}$+ $C_{(10)}$ cells (Fig. 1*B*). This experiment demonstrated an adaptation phenomenon where preconditioning with pROS induced resistance to a subsequent challenge.

Heterologous Adaptation, Resistance of H5V Cells to pROS Challenge following Preconditioning with H_2O_2—To compare the photoinduced adaptation phenomenon with previously described heterologous preconditioning based on H_2O_2 (34, 35), we examined the ability of H_2O_2 to induce resistance to a pROS challenge (Fig. 2*A*). As can be seen, preconditioning of H5V cells with increasing concentrations of H_2O_2 induced increased resistance to the pROS challenge with 5 μM WST11 reaching a plateau at 100 μM H_2O_2. Preconditioning alone led to cell death (0–30%) as indicated in the legend to Fig. 2. This result sug-

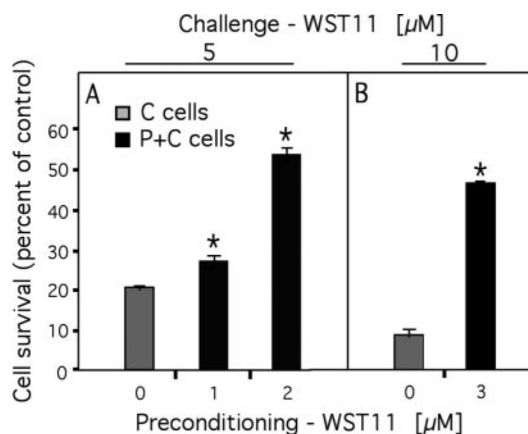

FIG. 1. **Homologous adaptation, resistance of H5V cells to pROS challenge following preconditioning with pROS.** H5V cells were preconditioned with WST11 at the indicated concentrations and light. The cells were challenged 24 h later with light + WST11 at 5 μM (*A*) or 10 μM (*B*). *Asterisks* (*) represent a significant difference of P + C cells (*black bars*) from the respective C cells (*gray bars*), $p < 0.05$.

gested that the adaptation of H5V cells to pROS challenge can be induced by homologous and heterologous oxidative stressors. The above experiments (Figs. 1 and 2*A*) suggested that the evolution of the adaptive phenotype requires an extended (24 h) time interval. Fig. 2*B* demonstrates the hypersensitivity of H5V ECs to pROS 30 min after preincubation with H_2O_2. This hypersensitivity is demonstrated even when utilizing pROS at LD_{50} (3 μM WST11 and light).

Preconditioning of H5V Cells with pROS Is Associated with Synthesis of Specific Proteins, hsp70 and hsp27—The extended time interval for evolution of adaptation demonstrated in Figs. 1 and 2 implies the involvement of protein synthesis in this process. Focusing on the homologous adaptation to the pROS challenge, we examined the possibility that the synthesis of specific proteins is induced during the 24-h time period following preconditioning. We therefore chose to monitor changes in the intracellular levels of two groups of proteins, HSPs and antioxidants following preconditioning ($P_{(3)}$ cells) (Fig. 3*A*). The induction of hsp70 expression was apparent 6 h after pROS generation, whereas hsp27 was induced later. By 16 h after photosensitization, both hsp70 and hsp27 reached a maximal level of induction that was maintained until 24 h after ROS generation. However, the protein levels of hsp90, hsp60, CuZn superoxide dismutase 1, and catalase were essentially unaffected during the same time period. Consistent with the suggested involvement of protein synthesis in the adaptation process, CHX, a general protein synthesis inhibitor was also able to block adaptation to pROS, (Fig. 3*B*). The preconditioning of H5V cells with pROS induced adaptation to a subsequent challenge in agreement with the results in Fig. 1. However, treatment with CHX during preconditioning suppressed the induction of resistance to subsequent pROS challenges at both 5 and 10 μM. The fact that CHX seems to reduce cytotoxicity to pROS challenge at 10 μM WST11 (Fig. 3*B*(*b*)) may be nonspecific and certainly not related to pROS adaptation. Whereas in P_0 (CHX) + C cells we tested the effect of CHX on the lethality of the pROS challenge, in P_3 (CHX) + C cells we tested the effect of CHX on the adaptation process, while only the latter being of relevance to our work. The bottom line is that this nonspecific effect did not impinge on the ability of CHX to inhibit adaptation when 10 μM WST11 pROS challenge was applied following preconditioning. Moreover, Fig. 3*C* demonstrated that CHX inhibited the synthesis of proteins induced during preconditioning, such as hsp70 and hsp27 but not hsp90, consistent with the results Fig. 3*A*.

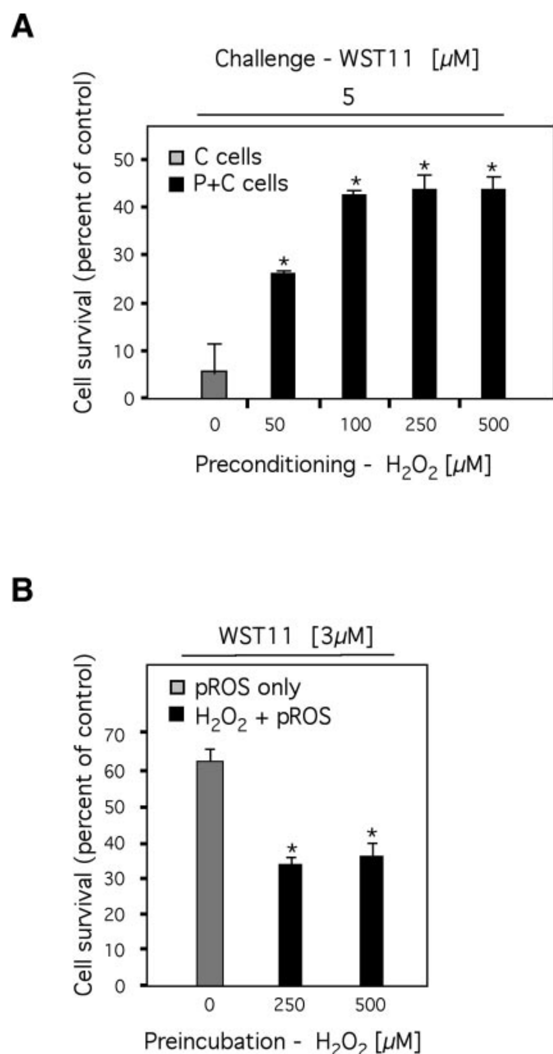

FIG. 2. **Heterologous adaptation, resistance of H5V cells to pROS challenge following preconditioning with H_2O_2.** *A*, H5V cells were preconditioned with H_2O_2 at the indicated concentrations and 24 h later challenged with light +5 μM WST11. *Asterisks* (*) represent a significant difference of P + C cells (*black bars*) from the respective C cells (*gray bars*), $p < 0.05$. Cell survival after preconditioning with each H_2O_2 concentration was as follows: 50 μM ~97%, 100 μM ~85%, 250 μM ~83%, and 500 μM ~72%. *B*, H5V cells were preincubated with H_2O_2 at the indicated concentrations and 30 min later were photosensitized with 3 μM of WST11.

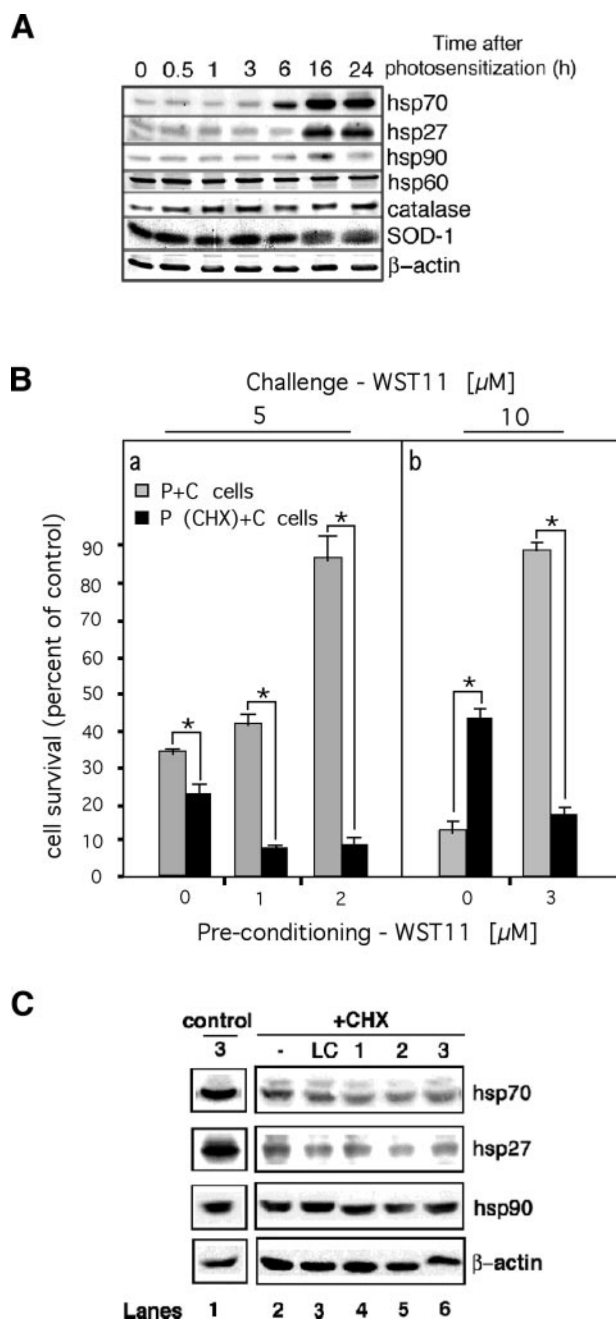

FIG. 3. **Preconditioning of H5V cells with pROS is associated with synthesis of specific proteins.** *A*, H5V cells were preconditioned with 3 μM WST11 + light, lysed at varying time intervals after treatment, and further analyzed by SDS-PAGE and Western blot analysis with the respective antibodies. *B*, H5V cells were preconditioned with the indicated concentrations of photosensitized WST11 in the absence (*black*) or presence (*gray*) of 0.1 μM CHX. 24 h later, P cells were challenged with photosensitized WST11 at 5 μM (*a*) or 10 μM (*b*). *Asterisks* (*) represent a significant difference of CHX-treated P + C cells from the respective P + C cells, $p < 0.05$. *C*, P cells prepared with the indicated concentration of photosensitized WST11 in the absence (*lane 1*) or presence (*lanes 2–6*) of CHX.

Expression of Specific HSPs and Antioxidant following Homologous Adaptation to pROS Challenge—To determine whether the induction of specific proteins during the period of preconditioning with pROS (Fig. 3*A*) can be correlated with homologous adaptation (Fig. 1), we next examined changes in these proteins at different steps of the homologous adaptation protocol (Fig. 4). In this experiment, P and C cells were harvested 24 h after the start of the experiment, and cell lysates were prepared. The Western blot results of $P_{(1),\ (2),\ \text{or}\ (3)}$ cells (Fig. 4, *lanes 4–6*), C cells $_{(5)\ \text{or}\ (10)}$ (*lanes 7* and *8*) and $P_{(1),\ (2),\ \text{or}\ (3)}$ + $C_{(5)\ \text{or}\ (10)}$ cells, respectively (*lanes 9–11*), as compared with controls (*lanes 1–3*) are presented.

It was found that hsp70 and hsp27 are strongly induced 24 h after ROS generation with different concentrations of WST11. Basal levels of hsp70 in controls (Fig. 4, *lanes 1–3*) gradually increased with increasing concentrations of photosensitized WST11 (*lanes 4–8*). Adapted $P_{(1),\ (2),\ \text{or}\ (3)}$ + $C_{(5)\ \text{or}\ (10)}$ cells (Fig. 4, *lanes 9–11*) exhibited higher hsp70 levels compared with $P_{(1),\ (2),\ \text{or}\ (3)}$ cells (*lanes 4–6*) but lower levels than non-adapted $C_{(5)\ \text{or}\ (10)}$ cells (*lanes 7* and *8*). Induction of hsp27 showed a similar pattern to that of hsp70 with a notable induction apparent in $P_{(3)}$ cells (Fig. 4, *lane 6*) and further increased with increasing concentrations of photosensitized WST11. In $P_{(1),\ (2)}$ + $C_{(5)}$ cells (Fig. 4, *lanes 9* and *10*) there were low levels of hsp27 similar to control levels. In contrast, adapted $P_{(3)}$ + $C_{(10)}$ cells (Fig. 4, *lane 11*) exhibited similar hsp27 levels to $P_{(3)}$ cells (*lane 6*) but 3 times lower hsp27 levels than in non-adapted $C_{(10)}$ cells (*lane 8*). In contrast to the above HSPs, hsp90 and hsp60 levels remained unchanged during

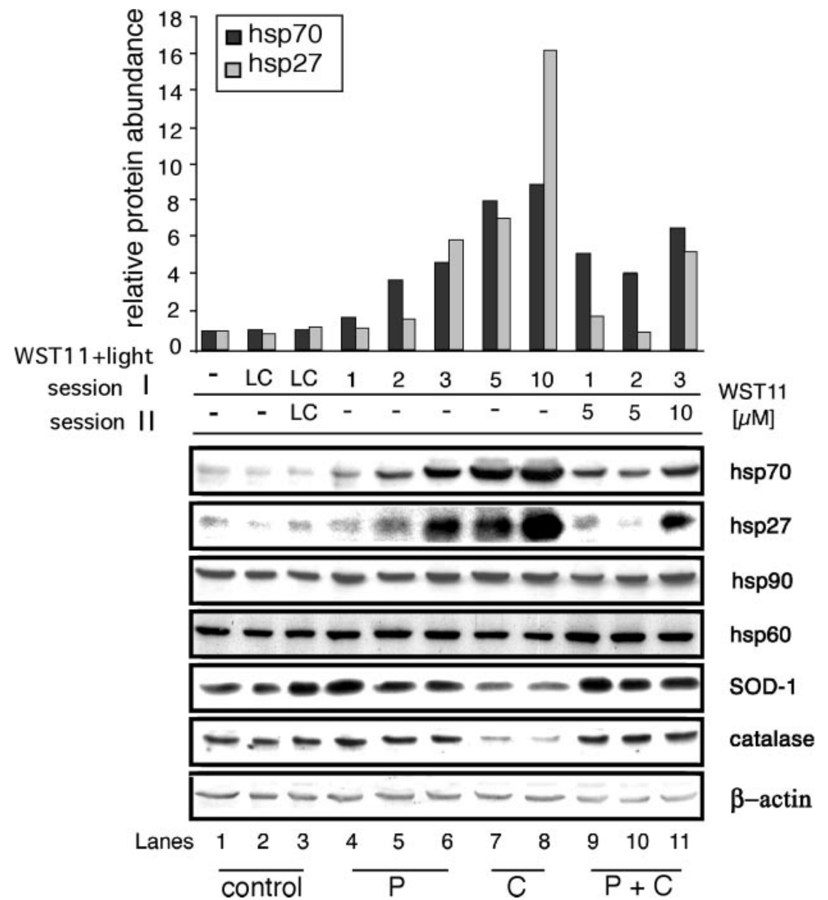

FIG. 4. **Expression of specific HSPs and antioxidant following homologous adaptation to pROS challenge.** H5V cells were treated once (*I*) or twice (*II*) with the indicated concentrations of WST11 + light, lysed 24 h later, and subjected to SDS-PAGE and Western blot analysis with the respective antibodies. *SOD-1*, CuZn superoxide dismutase. The *bar graph* represents the relative protein abundance to untreated control (−/−) values (normalized to β-actin) that was determined by densitometry for hsp70 (*black bars*) and hsp27 (*gray bars*). The untreated and light controls (*LC*) (*lanes 1–3*), P cells (*lanes 4–6*), C cells (*lanes 7–8*), and P + C cells (*lanes 9–11*) are presented.

these experiments. Interestingly, the levels of the antioxidant enzymes CuZn superoxide dismutase 1 and catalase were similar in adapted $P_{(1),\ (2),\ or\ (3)}$ + $C_{(5)\ or\ (10)}$ cells (Fig. 4, *lanes 9–11*), $P_{(1),\ (2),\ or\ (3)}$ cells (*lanes 4–6*), and control cells (*lanes 1–3*), although they were reduced (catalase more than CuZn superoxide dismutase 1) in non-adapted $C_{(5)\ or\ (10)}$ cells (*lanes 7–8*). In summary, it seems that the homologous adaptation process is associated with changes in cellular levels of specific proteins.

Involvement of p38 in Homologous Adaptation of H5V Cells to pROS—Previous observations in our laboratory have shown that in mouse melanoma cells, p38 is activated by photosensitization with Pd-bacteriochlorophyll-serine.[2] It was therefore presumed that pROS-induced changes in HSPs (Figs. 3 and 4) and p38 activation may be linked.

We first verified that photosensitization at increasing concentrations of WST11 also leads to increased phosphorylation of p38 in H5V cells, as determined 1-h postillumination (Fig. 5*A*). The consequent catalytic activity of p38 following the photosensitization of the cells with WST11 in $P_{(3)}$ cells was also determined via examination of MAPKAPK-2 phosphorylation by its only known activator, p38 (26, 36) (Fig. 5*B*). As expected, ROS generation was found to stimulate phosphorylation of MAPKAPK-2, which was sustained for ~6 h. The observed phosphorylation of MAPKAPK-2 by p38 activation was further validated by selective inhibition with SB, under conditions that were reported to be highly specific for p38 and not expected to effect other MAPKs as ERK and JNK (37–40). In this study, 10 μM SB was found sufficient to completely inhibit the catalytic activity of p38 (Fig. 5*C*). Furthermore, this particular concentration was reported to inhibit 95% of the relevant p38 isoforms in H5V (embryonic heart endothelial) cells (38, 39). To examine the possible link between p38 activation, homologous adaptation, and enhanced expression of hsp70 and hsp27 we first tested whether SB will interfere with the adaptation process. We found that homologous adaptation to pROS in H5V cells is significantly inhibited by 10 μM SB (Fig. 5*D*), consistent with the proposed role of p38 in this process. Because the 24-h preconditioning period seems to be essential for the induction of adaptation-specific proteins, the effect of SB on the expression of hsp70/hsp27 during the preconditioning period was also examined. As can be seen, SB inhibited the expression of hsp70 (Fig. 6*A*) and hsp27 (Fig. 6*B*) following pROS generation, correlating with the preconditioning levels relevant for the induction of each of these proteins in the course of homologous adaptation. The specificity of the process was further deduced from the finding that SB did not affect the levels of other proteins presented in Figs. 3 and 4. From each group of proteins, *i.e.* heat shock proteins and antioxidants, one representative was examined. We found that neither hsp90 (Fig. 6*C*) nor CuZn superoxide dismutase 1 (Fig. 6*D*) exhibited any change in level or pattern of expression, with or without SB202190. In summary, these results established a link between p38 activity and hsp70/hsp27 expression. These results also strongly connected p38 activation and the development of homologous adaptation to pROS in H5V cells.

DISCUSSION

This study demonstrates that the exposure of H5V heart mouse embryonic endothelial cells to WST11-based pROS induces adaptation to oxidative stress as manifested by resistance to subsequent ROS challenge and also by the induction of specific gene products likely to be involved in this process. The adaptation phenomenon is shown to be principally independent of the type of ROS used for preconditioning (Fig. 1, pROS or Fig. 2*A*, H_2O_2) and was defined as homologous (Fig. 1) or heterologous (Fig. 2*A*). Establishing the model for the study of

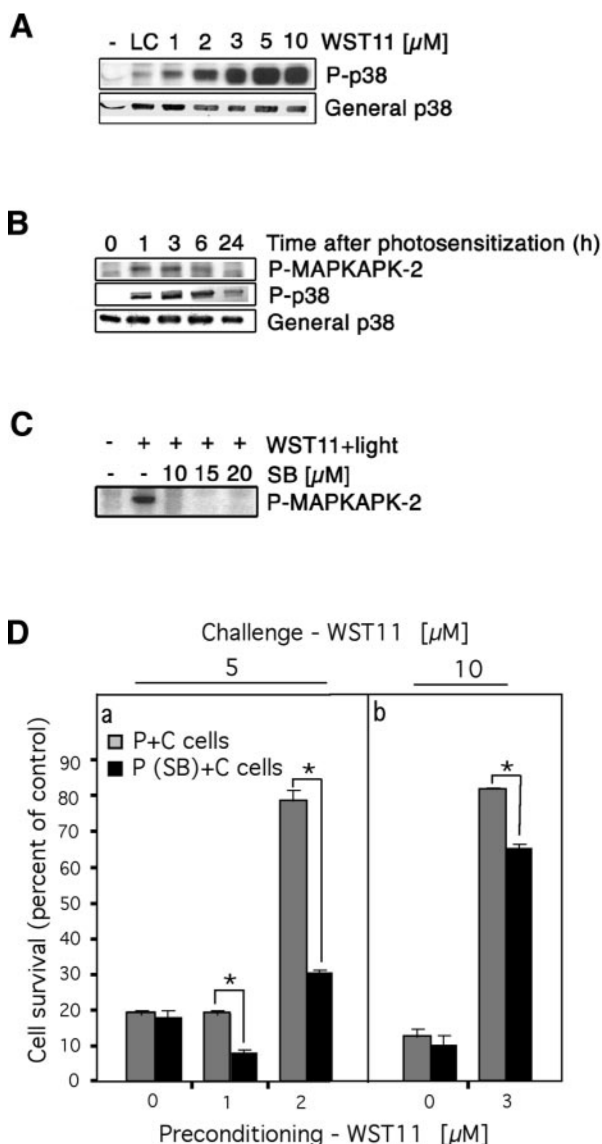

FIG. 5. **SB202190 inhibits p38 activity, homologous adaptation of H5V cells to pROS, and consequent expression of hsp70/hsp27.** *A*, H5V cells were subjected to photosensitization with the indicated concentrations of WST11 + light, lysed 1 h later, and subjected to SDS-PAGE and Western blot analysis with anti-phosphorylated-p38 (*P-p38*) and for protein abundance with anti-general p38 antibodies. Untreated (−) and light controls (*LC*) are also presented. *B*, H5V cells were subjected to photosensitization with 3 μM WST11 + light, lysed at indicated times after treatment, and lysates were subjected to SDS-PAGE and Western blot analysis. The catalytic activity of p38 was determined by using anti-phosphorylated-MAPKAPK-2 antibodies. Anti-phosphorylated-p38 and anti-general p38 antibodies were used for detection of phosphorylation and abundance of p38, respectively. *C*, H5V cells were subjected to photosensitization with 3 μM WST11 + light without or with the indicated concentrations of SB, lysed 1 h after treatment, and further analyzed by SDS-PAGE and Western blot analysis. The catalytic activity of p38 was determined with anti-phosphorylated-MAPKAPK-2 antibodies. *D*, H5V cells were preconditioned with the indicated concentrations of WST11 + light in the absence (*black bars*) or presence (*gray bars*) of SB 10 μM. 24 h later, P cells were challenged with light + WST11 5 μM (*a*) or 10 μM (*b*). *Asterisks* (*) represent a significant difference of SB-treated P + C cells from the respective P + C cells, $p < 0.05$.

homologous adaptation to pROS confirmed previous findings involving other forms of stress (3, 41) that the induction process is time-dependent to allow the synthesis of essential protective proteins (Fig. 3). Among proteins induced by preconditioning, we identified specific proteins such as the inducible form of hsp70 and the small HSP, hsp27, and found other HSPs and antioxidants that were not affected by the process (Figs. 3 and 4). The HSPs induced are proposed to be important participants in signaling and maintenance of the oxidative-stress-adapted phenotype. The dependence of the adaptive phenotype upon the expression of hsp70 and hsp27 is further suggested by Figs. 1 and 2. The evolution of adaptation to pROS challenge after preconditioning with either H_2O_2 (Fig. 2*A*) or pROS (Fig. 1*A*) requires ~24 h in contrast to the hypersensitivity to pROS observed 30 min after preincubation with H_2O_2 (Fig. 2*B*). This correlates with the time of selective protein synthesis activation of the above HSPs (>6 h).

Moreover, the results indicate that adaptation of H5V cells to pROS-induced oxidative stress is associated with the activation of p38, an enzyme that evidently plays a role in hsp70/hsp27 induction as demonstrated by the interference of these processes using the specific p38 inhibitor SB202190 (Figs. 5 and 6). Of the four known isoforms of p38, only p38 and p38β are relevant to H5V heart endothelial cells, both are inhibited by SB202190 (39). Whether one or both of these enzymes maybe involved in the process remains to be examined.

It has been described previously that hsp70 is associated with thermotolerance (1) and was also shown to be induced by photosensitization (42, 43) mainly by photosensitizers (chlorin- and purpurin-based) that preferentially localize in the lysosomes (44, 45). Interestingly, the linkage between hsp27 expression and the induction of the resistant phenotype by adaptation are in agreement with constitutive expression of this HSP in photofrin-resistant HT29-P14 cells (46). In addition to enhanced hsp27 expression upon oxidative stress, this protein has been shown to be acutely phosphorylated by the p38-mediated pathway (47). However, stress-induced p38 activation is not always associated with phosphorylation of the small HSPs, suggesting that cytoprotection may be mediated by an increase in cellular levels of these proteins (48).

It should be noted that the expression of hsp70 and 27 in the above studies was investigated following a single photosensitization step conducted during *in vitro* PDT under lethal (44, 46) or sublethal conditions (42, 43). However, to examine the role of HSPs in the process of adaptation as studied here, their expression levels were monitored within the time interval spanning the preconditioning step (that induces their expression) and the subsequent photodynamic challenge when their protective effects were displayed. Moreover, as WST11-PDT is an anti-vascular therapy, we studied adaptation using endothelial cells, whereas other studies dealt with the effect of pROS on tumor cells. It appeared that the homologous adaptation of H5V cells to pROS, according to the described protocol, showed a certain degree of selectivity, whereas other putative markers (hsp90, hsp60) remained unchanged. We chose to examine the hsp90 isoforms as they are among the most abundant of cytoplasmic proteins associated with plasma membrane signaling pathways including stress-induced pathways (17). In ECs, endothelial nitric oxide synthetase is a client protein of hsp90 (49), and its acute activation by pROS in H5V cells was recently shown in our laboratory (50). Because hsp90 modulation of endothelial nitric oxide synthetase activity is achieved by recruitment of existing cytoplasmic hsp90 (17), photosensitized activation of this enzyme in ECs may not require the enhancement of hsp90 levels. Consideration of hsp60 as a marker for the adaptive process studied here was related to its induction by photosensitization with Photofrin, a sensitizer that preaccumulates in the mitochondria (51). The fact that the hsp60 level was unchanged in our study may suggest that WST11 does not localize to mitochondria.

The capacity of ECs to adapt to oxidative stress can also be observed by their ability to regulate the levels of stress proteins

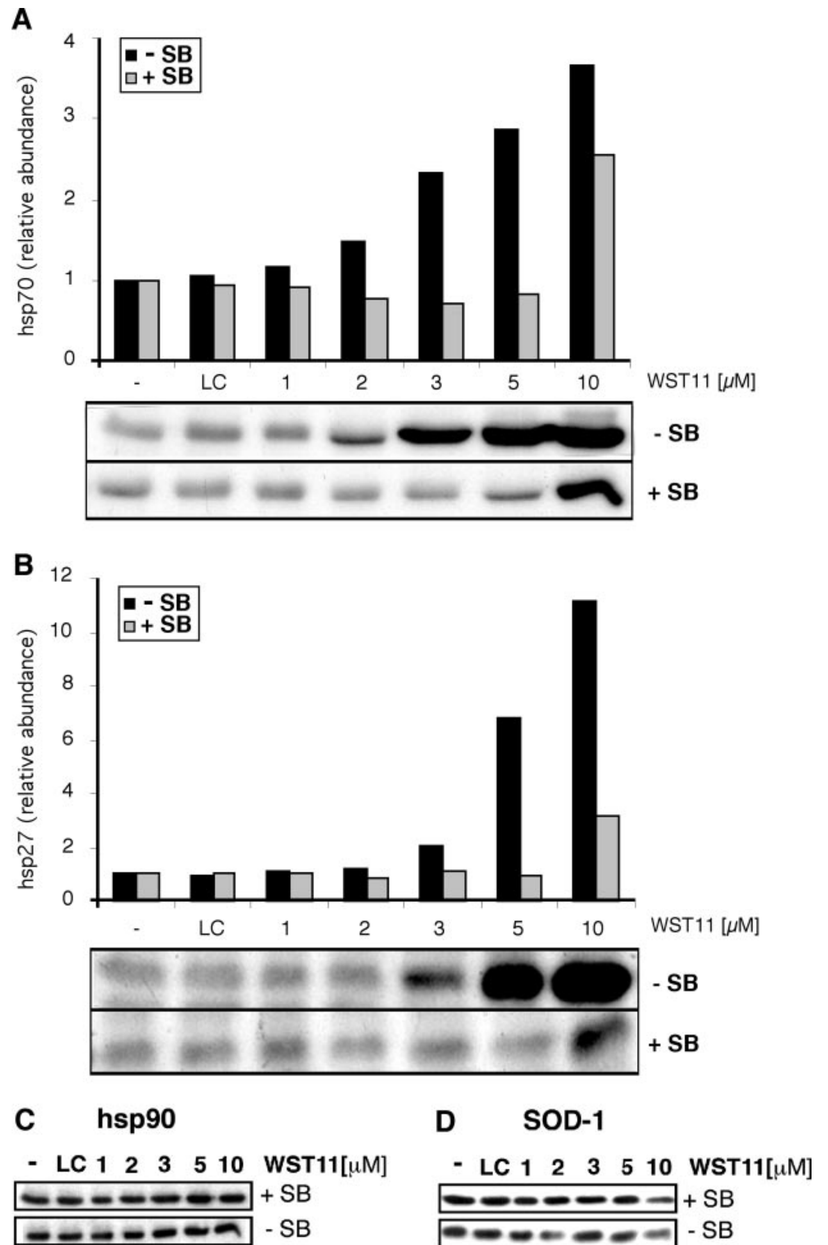

FIG. 6. **Inhibition of pROS-induced hsp70 and hsp27 expression in H5V cells using SB202190.** H5V cells were subjected to photosensitization with the indicated concentrations of WST11 + light in the absence or presence of 10 μM SB. Cells were lysed 24 h later and subjected to SDS-PAGE and Western blot analysis with anti-hsp70 (*A*) and anti-hsp27 (*B*). The relative abundance of hsp70/hsp27 to untreated control values (normalized to β-actin) was determined by densitometry. The absence or presence of SB is indicated by *black* or *gray bars*, respectively. Untreated cells (−) and light controls (*LC*) are also presented. As a control for the selective action of SB, reprobing with anti-hsp90 (*C*) and anti-CuZn superoxide dismutase 1 (*D*, *SOD-1*) exhibited no change in the levels and pattern of expression of these proteins.

in response to repeated challenges (1). Such a process was indeed noted here (Fig. 4), where the levels of induced HSPs in adapted P + C cells (*lanes 9–11*) were higher than in the respective P cells (*lanes 4–6*) but lower than in non-adapted C cells (*lanes 7* and *8*). This observation demonstrates that the synthesis of HSPs in P cells, followed by reprobing with a pROS challenge, may be autodown-regulated by preconditioning-induced levels. Autodown-regulation during a second stress encounter is a hallmark of adaptation, as in the case of thermotolerance (1). HSPs, as part of their role in maintaining translational and protein integrity, are among the first to be translated after cells suffer stress-induced translational arrest. This translational arrest is shortened in adapted cells, probably because of the presence of HSPs that as a consequence, autodown-regulate their own levels. We also examined the possible involvement of cellular enzymatic antioxidants in pROS-induced adaptation to oxidative stress and observed no induction of catalase nor CuZn superoxide dismutase 1. Yet we observed a decrease in these antioxidant levels in non-adapted C cells (Fig. 4). Interestingly, in adapted P + C cells, catalase and CuZn superoxide dismutase 1 levels were maintained possibly because of protection by HSPs already present in preconditioned cells at the time of the challenge. This interpretation does not exclude the possibility of changes in the catalytic activity of these enzymes by oxidative stress (21).

The identity of the pathway(s) involved in the regulation of adaptation to pROS is presently unknown. General transcription pathways for various HSPs (as hsp70 and hsp27) mostly involve heat shock transcription factor 1, which binds to the promoter element of the gene during stress (1, 52, 53). It was previously suggested that heat shock transcription factor 1 is the target of more than one member of the MAP kinase family (ERK, JNK, and p38) (53, 54). This could be a potential pathway by which pROS-activated p38 (Fig. 5*A*) contributes to HSP-mediated homologous adaptation of ECs.

In summary, this study demonstrates that ECs are capable of adaptation to pROS induced by photosensitized WST11. We also demonstrated that the associated expression pattern of hsp70/hsp27 coincides with homologous adaptation to pROS and that homologous adaptation and the associated expression of hsp70/hsp27 are linked to p38 activation. Although the specific p38 inhibitor (SB202190) abolished hsp70/hsp27 induction

in course of homologous adaptation to pROS, it only partially inhibited the adaptation process itself. This may suggest the involvement of additional pathways in this complex mechanism. Nevertheless, our observations form the basis for further studies using controlled photosensitization as a tool for the elucidation of adaptation mechanisms to pROS and other types of oxidative stress.

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