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trans-Resveratrol Protects Ischemic PC12 Cells by Inhibiting the Hypoxia Associated Transcription Factors and Increasing the Levels of Antioxidant Defense Enzymes

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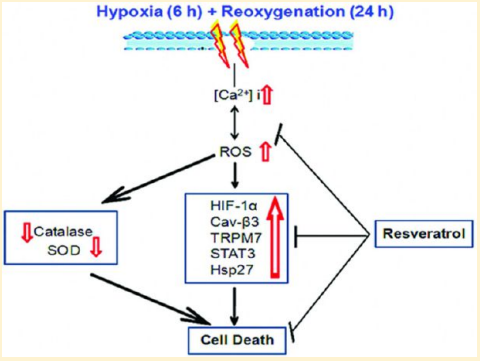
ABSTRACT: An in vitro model of ischemic cerebral stroke [oxygen-glucose deprivation (OGD) for 6 h followed by 24 h reoxygenation (R)] with PC12 cells increases Ca^{2+} influx by upregulating native L-type Ca^{2+} channels and reactive oxygen species (ROS) generation. This reactive oxygen species generation and increase in intracellular Ca^{2+} triggers the expression of hypoxic homeostasis transcription factors such as hypoxia induced factor-1 alpha (HIF-1α), Cav-beta 3 (Cav β3), signal transducer and activator of transcription 3 (STAT3), heat shock protein 27 (hsp-27), and cationic channel transient receptor potential melastatin 7 (TRPM7). OGD insulted PC12 cells were subjected to biologically safe doses (5, 10, and 25 μM) of *trans*-resveratrol in three different treatment groups: 24 h prior to OGD (pre-treatment); 24 h post OGD (post-treatment); and from 24 h before OGD to end of reoxygenation period (whole-treatment). Here, we demonstrated that OGD-R-induced neuronal injury/death is by reactive oxygen species generation, increase in intracellular calcium levels, and decrease in antioxidant defense enzymes. *trans*-Resveratrol increases the viability of OGD-R insulted PC12 cells, which was assessed by using MTT, NRU, and LDH release assay. In addition, *trans*-resveratrol significantly decreases reactive oxygen species generation, intracellular Ca^{2+} levels, and hypoxia associated transcription factors and also increases the level of antioxidant defense enzymes. Our data shows that the whole-treatment group of *trans*-resveratrol is most efficient in decreasing hypoxia induced cell death through its antioxidant properties.



KEYWORDS: *PC12 cells, OGD, intracellular calcium, ROS, trans-resveratrol*

Oxygen availability is important for cellular metabolism. However, inadequate oxygen (hypoxia) triggers a multifaceted cellular response that leads to cellular death. The cellular chain of events is initiated by increased calcium influx triggering the activation of proteases, phospholipases, and the formation of free radicals.[1,2] Hypoxia triggers an increase in the generation of reactive oxygen species (ROS), inducing a significant activation of signal transduction cascades to cause an increase in cytosolic Ca^{2+} concentration.[3,4] Ca^{2+} and ROS signaling systems are intimately integrated; components of ROS homeostasis dependent on Ca^{2+} regulation might influence intracellular redox balance, and vice versa.[5] Failure of adaptive homeostatic mechanisms leads to persistent hypoxia/ischemia and neuronal death through apoptosis or necrosis (among many other pathways).[6] In addition to increased Ca^{2+} signaling, Yuan et al.[7] reported that exposing PC12 cells to hypoxia leads to increased expression of the HIF-1α protein, a major oxygen homeostasis regulator. Under normoxic conditions, HIF-1α is rapidly degraded by the proteasome.[8] However, under hypoxic conditions, HIF-1α is stabilized and permits the activation of genes essential for cellular adaptation to low oxygen conditions. These genes include STAT3, hsp-27, Cav β3, native L-type Ca^{2+} channels, vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporter-1. Cav β subunits are cytoplasmic proteins that strongly regulate Cav channels through direct interaction with pore-forming α1 subunits.[9,10] STAT3 protects cell against hypoxia by inducing the expression of antioxidant defense enzymes such as superoxide dismutase.[11]

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) strongly protects PC12 cells from hypoxia induced oxidative cell death.[12] This neuroprotection works through the scavenging of free radicals and also through modulating proinflammatory mediators and cytokines via activation of mTOR signaling pathway and the inhibition of the transcriptional factors such as NF-κB and activator protein-1 (AP-1). These events help in restoration of optimal cell communication.[13,14] We have previously reported that oxygen-glucose deprivation (OGD) increases the expression of HIF-1α which induces apoptosis in PC12 cells.[15] The in vitro model using a rat pheochromocytoma cell line (PC12 cells) is well established in our lab.[16] This model is unique as this cell type shares a number of characteristics with the oxygen-sensing type cells.[17]

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Here we report that *trans*-resveratrol, a well-known phenolic compound with many biologic activities, opposes the toxic effects of hypoxia by its direct or indirect effects in PC12 cells.[18−20] In the present study, we determined the time and dose response of *trans*-resveratrol and its mechanistic modulation of signaling in an in vitro ischemic model of oxygen-glucose deprivation followed by reoxygenation.

RESULTS AND DISCUSSION

Estimation of Noncytotoxic Doses of *trans*-Resveratrol in PC12 Cells. Biologically safe concentrations of *trans*-resveratrol were determined. Different concentrations (1−1000 μM) of *trans*-resveratrol were used to treat PC12 cells for 24−96 h and subsequently assessed by MTT assay. Highlights of the results are summarized in Figure 2. Higher concentrations

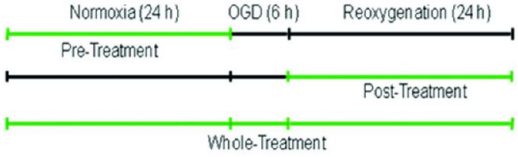

Figure 1. Diagrammatic representation of treatment schedule.

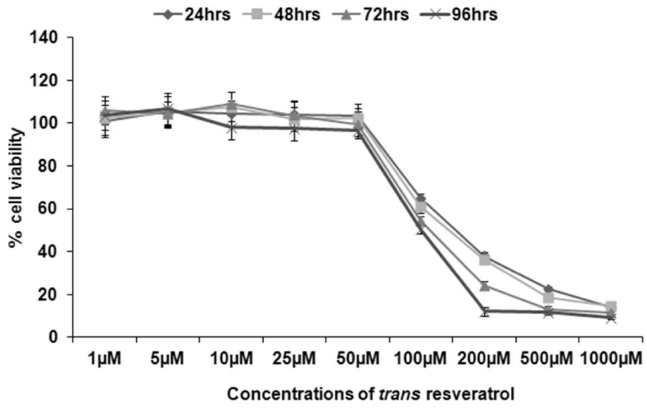

Figure 2. Cytoprotective doses of *trans*-resveratrol. Effect on percent cell viability in PC12 cells by MTT assay following (1−1000 μM) concentrations of *trans*-resveratrol for various time intervals (24−96 h). Values are mean ± SEM of three experiments each carried out in triplicate.

of 100, 200, 500, and 1000 μM *trans*-resveratrol have shown significant loss in percent cell viability (35 ± 2.1%, 42 ± 2.8%, 78 ± 3.9%, and 86 ± 4.4% of control, respectively) even at 24 h exposure. The reduction in cell viability was continued in further exposures and reached 50 ± 2.4%, 88 ± 5.2%, 89 ± 6.1%, and 91 ± 7.6% of control, respectively, at 96 h. PC12 cells treated with 50 μM and lower concentrations of *trans*-resveratrol were found to be 100% viable at all the time intervals (24−96 h) (Figure 2). The concentrations selected for the study were 5, 10, and 25 μM.

Cytoprotective Effect of *trans*-Resveratrol (MTT). Cell viability was assessed immediately after the OGD-R. The viability of cells decreased significantly after 6 h of OGD and 24 h of reoxygenation when compared to normoxia control. At the start of experiment, under normoxia conditions (0 h), the percent cell viability was measured 97.0 ± 0.6%, whereas after OGD-R the viability was measured 60.0 ± 0.6%. A statistically significant increase in cell viability was observed in the whole-treatment group (83.8 ± 4.9%, 90.2 ± 6.2%, and 94.9 ± 7.1%) when compared to control at 5, 10, and 25 μM *trans*-resveratrol, respectively. A similar trend of *trans*-resveratrol mediated protection was also observed as in the case of the post-treatment group, though the magnitude of protection was comparatively lower (Figure 3a). The *trans*-resveratrol

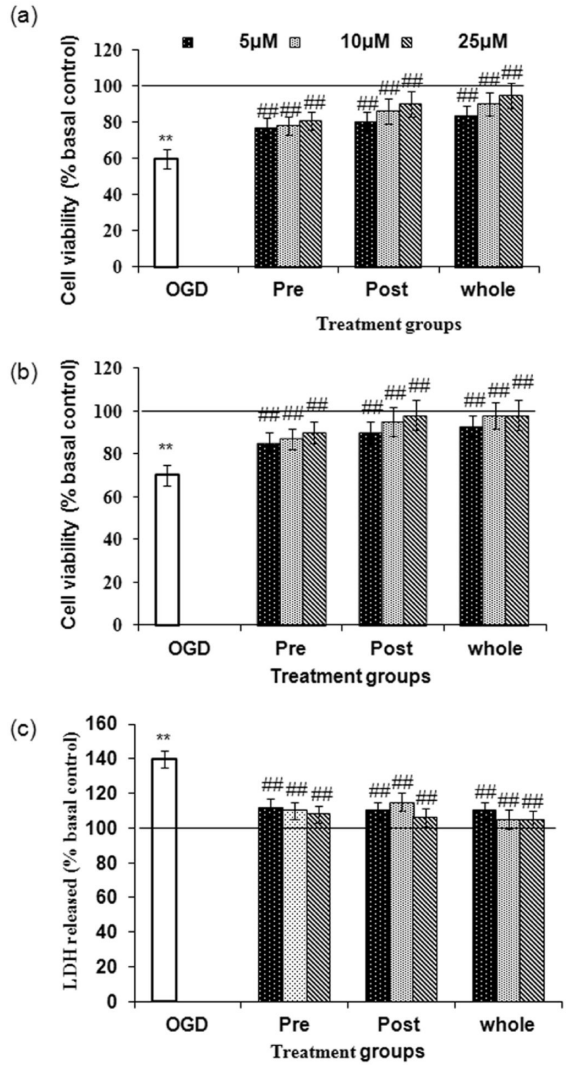

Figure 3. Protective potential of *trans*-resveratrol (5, 10, and 25 μM) on percent cell viability: (a) MTT assay, (b) NRU assay, and (c) LDH assay in PC12 cells following the 6 h OGD and 24 h reoxygenation. $^{**}P < 0.01$ when OGD is compared with normoxia control; $^{\#\#}P < 0.01$ when treatment groups are compared with OGD. Values are mean ± SEM of three experiments each carried out in triplicate.

mediated increase in percent cell viability was statistically significant, when compared with OGD-R exposed group and the protection was concentration dependent. All the concentrations in the pretreatment schedule given 24 h prior to OGD-R were found to be less effective.

Cytoprotective Effect of *trans*-Resveratrol (NRU). The cytoprotective potential of *trans*-resveratrol against OGD-R in PC12 cells by neutral red uptake (NRU) assay is shown in Figure 3b. Cell viability decreased significantly after 6 h of OGD and 24 h of reoxygenation to 70 ± 4.9% when compared to normal control cells. All the treatment groups of *trans*-resveratrol show statistically significant increases in cell viability

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with all three concentrations, 5, 10, and 25 μM. At each concentration assessed, the magnitude of protection was highest in whole-treatment group.

Lactate Dehydrogenase (LDH) Release. A significant increase in LDH release was observed in PC12 cells subjected to OGD (140 ± 4.9%) in comparison to normoxia control group (Figure 3c). The same pattern of reduction in the LDH release could be recorded following all pre-, post-, and whole-treatment of *trans*-resveratrol. A pretreatment of *trans*-resveratrol (5, 10, and 25 μM) brought down the LDH release to 112 ± 5.2%, 110 ± 4.9%, and 108 ± 5% of normoxia control, respectively. Such reduction in LDH release was statistically significant to the increase in LDH release by OGD insult. The values for post-treatment were (110 ± 5.1, 115 ± 5.2 and 106 ± 5.3%) following 5, 10, and 25 μM, respectively. The whole-treatment of *trans*-resveratrol brings down the values to (110 ± 4.9%, 105 ± 5.4%, and 105 ± 4.9%) following 5, 10, and 25 μM, respectively.

Intracellular Ca^{2+} Measurements. Increase in $[Ca^{2+}]_i$ after excitotoxic insults has been linked to free radical production, which leads to cell death. Under our experimental setup, PC12 cells of the OGD group have shown a statistically significant increase in the intracellular calcium levels (343.6 ± 16.8%) compared to the normoxia control group. All the treatment groups of *trans*-resveratrol have shown a statistically significant reduction in $[Ca^{2+}]_i$ levels; however, the reduced levels were even significantly higher than those in the normoxia control group. Among the treatment groups, the whole-treatment group has shown maximum reduction of intracellular $[Ca^{2+}]_i$ levels in a dose dependent manner (Figure 4). The highest concentration (25 μM) used in the whole-treatment group was found to be most effective in reducing the levels of intracellular $[Ca^{2+}]_i$, that is, 143 ± 12% of normoxia control.

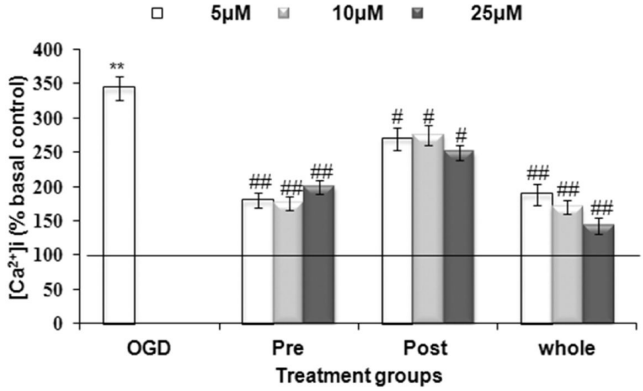

Figure 4. Intracellular calcium levels in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of 5, 10, and 25 μM concentrations of *trans*-resveratrol. $^{**}P < 0.01$ when OGD is compared with normoxia control, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ when treatment groups are compared with OGD. Values are mean ± SEM of three experiments each carried out in triplicate.

Reactive Oxygen Species Generation. Reactive oxygen species generation in PC12 cells was measured by using DCF-DA dye with fluorescence microscopy. Significant induction in reactive oxygen species production (6.36-fold) was recorded in hypoxic cells when compared to normoxia control (Figure 5). Although the reactive oxygen species generation was reduced significantly (3.63-, 3.14-, and 2.33-fold) following pretreatment with *trans*-resveratrol at concentrations of 5, 10, and 25 μM,

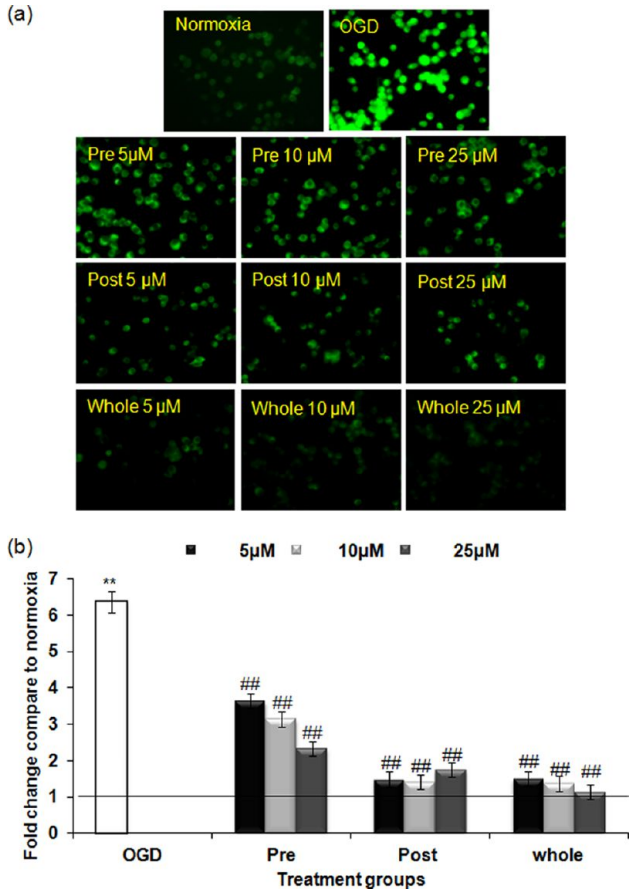

Figure 5. (a) Representative microphotographs showing 6 h OGD and 24 h reoxygenation-induced reactive oxygen species (ROS) generation and effect of 5, 10, and 25 μM concentrations of *trans*-resveratrol in PC12 cells. Images were captured via a Nikon phase contrast fluorescence microscope (model 80i) with an attached 12.7 megapixel Nikon DS-Ri1 digital CCD cool camera. Values are mean ± SEM of three experiments each carried out in triplicate. (b) Fold change in reactive oxygen species generation. Quantification of fluorescence was done using image analysis software Leica Q-win 500, and data expressed in-fold of unexposed control. $^{**}P < 0.01$ when OGD is compared with normoxia control; $^{\#\#}P < 0.01$ when treatment groups are compared with OGD. Values are mean ± SEM of three experiments each carried out in triplicate.

respectively, values were still higher than those for the normoxia control. Post- and whole-treatment exhibited a drastic reduction in OGD induced reactive oxygen species generation and brought down the values closer to the normoxia control. The highest concentration of *trans*-resveratrol (25 μM) of the whole-treatment group was most effective and brought down the value of reactive oxygen species parallel to the normoxia control (1.15-fold).

Superoxide Dismutase (SOD) Activity. The modulatory effect of *trans*-resveratrol on superoxide dismutase activity in PC12 cells receiving OGD insult is summarized in Figure 6a. A significant decrease in the SOD activity was observed in the cells following 6 h OGD (70.6 ± 3.5%) when compared to normoxia control. Post- and whole-treatment of *trans*-resveratrol was found to be statistically significant protective at all the concentrations used. In general, the highest concentration of *trans*-resveratrol (25 μM) have shown the best restoration of SOD levels, that is, 85.15 ± 3.31%, 91.74 ±

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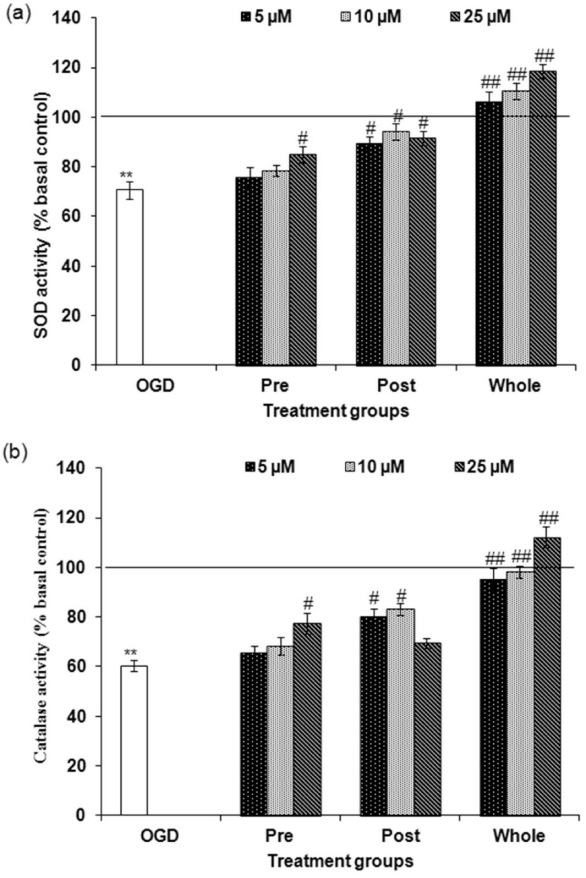

Figure 6. (a) Superoxide dismutase activity and (b) catalase activity in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of 5, 10, and 25 μM concentrations of *trans*-resveratrol. $^{**}P < 0.01$ when OGD is compared with normoxia control, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ when treatment groups are compared with OGD. Values are mean ± SEM of three experiments each carried out in triplicate.

2.87%, and 118.5 ± 2.93% of control in pre-, post-, and whole-treatment groups, respectively.

Catalase Activity. Following OGD, a significant decrease in the overall activity of catalase was measured (60.4 ± 2.35%) compared to normoxia control. The pretreatment schedule showed a concentration dependent significant restoration in catalase activity, (65.75 ± 2.67%, 68.21 ± 3.56%, and 77.54 ± 4.3%) compared to normoxia control at 5, 10, and 25 μM, respectively. Increase in catalase activity was observed with 10 μM of post-treatment (83.28 ± 2.45%) when compared with normoxia control (Figure 6b). Moreover, a concentration dependent restoration in catalase activity was observed in the whole-treatment group that was maximum with 25 μM (112.14 ± 4.23%) when compared with normoxia control.

Transcriptional Changes. OGD-reoxygenation induced changes in mRNA expression for HIF-1α, Cav β3, TRPM7, STAT 3, and hsp-27, which are involved in various aspects related to hypoxia and intracellular calcium, and the protective potential of *trans*-resveratrol (Figure 7). Based on performance, only the 25 μM concentration of *trans*-resveratrol was selected in the expression studies. Since OGD influences the expression of GAPDH, hypoxanthine phosphoribosyltransferase (HPRT) was used as internal control (a housekeeping gene) to normalize the data against.

Expression for HIF-1α. OGD significantly increased the expression of the HIF-1α gene (990.3 ± 8.8-fold) when compared to normal control cells. A significant decrease in the expression of HIF-1α was observed in all the treatment schedules. Pre- and post-treatment exhibited a drastic reduction in OGD induced mRNA expression of the HIF-1α gene (514.2 ± 5.8- and 125.6 ± 4.3-fold) and brought down the values closer to those of the normoxia control. However, the whole-treatment group shows the best recovery, where maximum reduction in mRNA expression was recorded when compared to normoxia control (103.2 ± 4.3-fold; Figure 7a).

Expression for Cav β3. Post- and whole-treatment groups showed a statistically significant and similar magnitude of recovery, that is, 2.2 ± 0.1- and 2.1 ± 0.2-fold, respectively. However, there was a nonsignificant recovery of Cav β3 in the pre-treatment group (Figure 7b).

Expression for TRPM7. Significant changes in mRNA levels for TRPM7 in PC12 cells following OGD (3.36 ± 0.25-fold) when compared to normal control cells (Figure 7c). Interestingly, pre-treatment was not found to be very effective (2.2 ± 0.25-fold) in the study while post- and whole-treatment were able to bring down the levels of mRNA for TRPM7 significantly near to those of normoxia under our experimental conditions (1.33 ± 0.15- and 1.43 ± 0.20-fold, respectively).

Expression for STAT3. OGD-reoxygenation increased the expression of STAT3. A statistically significant increase in the expression of STAT3 was recorded in the OGD group (3 ± 0.1-fold) of the normoxia control (Figure 7d). Whole-treatment was found to be most effective in reducing the levels of mRNA in PC12 cells to 1.26 ± 0.1-fold followed by post-treatment (1.5 ± 0.1-fold) and then pre-treatment (2.2 ± 0.17-fold).

Expression for hsp-27. OGD insult was found to cause a significant increase in the mRNA expression of hsp-27 in PC12 cells (6.83 ± 0.3-fold) when compared to normal control cells. However, treatment specific changes were observed when cells were exposed to pre-treatment (3.9 ± 0.2-fold), post-treatment (2.2 ± 0.2-fold), and whole-treatment (1.9 ± 0.2-fold) (Figure 7e). Whole-treatment was most effective in bringing the values near to those of the normoxia control.

Translational Changes. Results of alterations in the expression of maker proteins associated with ischemic insult following OGD-reoxygenation and *trans*-resveratrol treatment assessed by Western blot are presented in Figure 8.

Expression of HIF-1α Protein. At the translational level, we observed similar trends as in transcriptional outcomes for HIF-1α protein expression but the values were lower. Densitometry analysis shows a highly significant increase in HIF-1α protein in the OGD group (≈25-fold) of the normoxia control. Interestingly, pre-treatment was found to be less effective (15.1-fold) than post- and whole-treatment which were strongly effective and able to bring down the levels of HIF-1α protein significantly near to those of control under our experimental conditions (7- and 4.33-fold, respectively).

Expression for Cav β3 Protein. OGD treatment increased the expression of Cav β3 approximately 2.6-fold of the normoxia control. Though a reduction in expression following pre-treatment of *trans*-resveratrol was observed, it was insignificant, while a significant decrease in the expression of Cav β3 protein was recorded in post- and whole-treatment (1.44- and 1.64-fold, respectively) of normoxia control.

Expression for TRPM7 Protein. A statistically significant rise in the TRPM7 protein expression was recorded in the OGD group (≈3.6-fold) of the normoxia control. A significant decrease in expression was observed in all the treatment schedules of *trans*-resveratrol with the best performance of

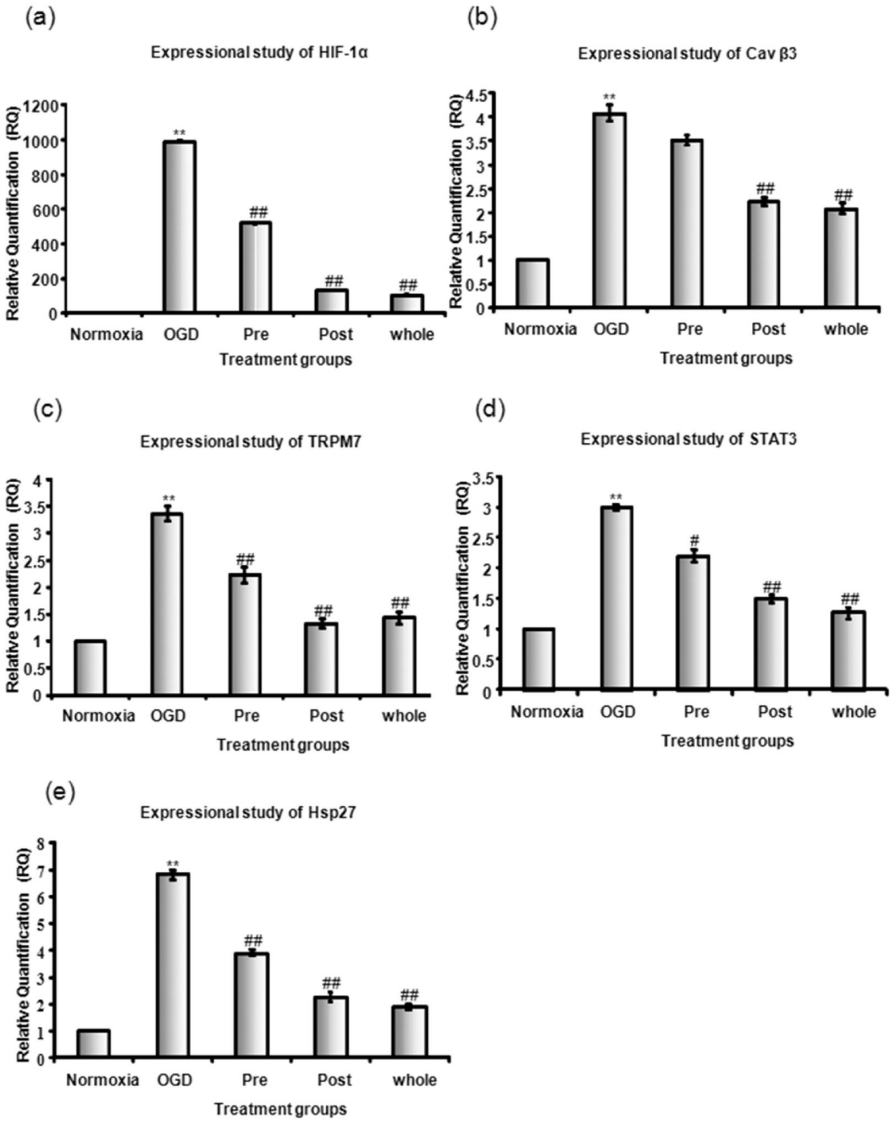

Figure 7. Relative quantification of-fold induction in the expression of (a) HIF-1α, (b) Cavβ 3, (c) TRPM7, (d) Stat3, and (e) hsp-27 using real time PCR in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of 25 μM treatment of *trans*-resveratrol. HPRT was used as internal control to normalize the data. Quantitative real time PCR (RT-PCR^q) was performed in triplicate using SYBR Green dye and an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). $^{**}P < 0.01$ when OGD is compared with normoxia control, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ when treatment groups are compared with OGD. Values are mean ± SEM of three experiments each carried out in triplicate.

post-treatment, where 1.4-fold reduction in the expression was observed when compared to the OGD group.

Expression for STAT3 Protein. A significant increase in STAT3 protein in the OGD group (≈2.3-fold) of the normoxia control was recorded. Interestingly, pre-treatment was not found to be much effective (2-fold) in the study while post- and whole-treatment were able to bring down the levels of STAT3 protein significantly near to those of control under our experimental conditions (1.29- and 1-fold, respectively).

Expression for hsp-27 Protein. A statistically significant rise in the hsp-27 protein expression was recorded in the OGD group (≈2.2-fold) of normoxia control. Though a reduction in expression following pre-treatment of *trans*-resveratrol was observed, it was insignificant, while a significant decrease in the expression of hsp-27 protein was recorded in post- and whole-treatment (1.53- and 1.35-fold, respectively) of normoxia control.

Numerous experimental observations have led to the hypothesis that ischemia-induced neuronal damage results from a chain of pathological events that involve cytotoxicity, oxidative stress, apoptosis, increase in intracellular calcium $[Ca^{2+}]_i$, and deregulation of genes whose expression promotes neurological dysfunction.[21−24] In the present study, we observed that OGD and reoxygenation result in the loss of cell viability, increase in LDH and reactive oxygen species production, and, subsequently, increase in intracellular calcium levels in PC12 cells. The results suggest that a cytosolic oxidant signal triggers an increase in $[Ca^{2+}]_i$ during hypoxia and the increase in oxidant signaling can be attenuated by an antioxidant which acts by reducing thiol groups and enhancing reactive oxygen species scavenging.[3]

trans-Resveratrol is present in red wine and is considered to be the substance responsible for lowering incidence of coronary heart disease among regular consumers of wine. In addition, several in vitro and in vivo studies have attributed this beneficial

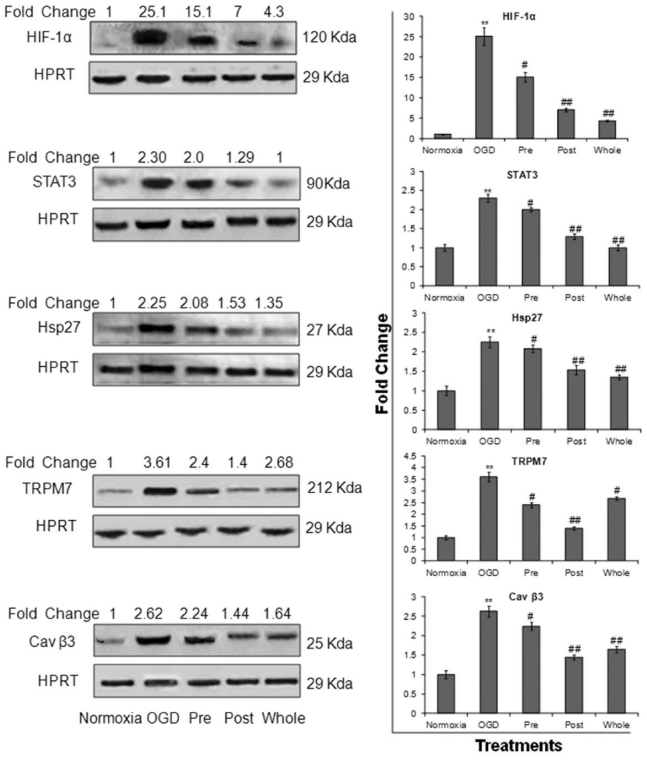

Figure 8. Alterations in the expression of proteins HIF-1α, Cav β3, TRPM7, STAT3, and hsp-27 using Western blot analysis in PC12 cells following OGD of 6 h and reoxygenation of 24 h and effect of 25 μM treatment of *trans*-resveratrol. HPRT was used as internal control to normalize the data. Quantification was done using a Gel Documentation system (Alpha Innotech) with the help of Alpha Ease FC StandAlone V.4.0 software. $^{**}P < 0.01$ when OGD is compared with normoxia control, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ when treatment groups are compared with OGD. Values are mean ± SEM of three experiments each carried out in triplicate.

effect of *trans*-resveratrol to its potent antioxidant activity. We studied the neuroprotective effect of *trans*-resveratrol using an in vitro ischemic model of PC12 cell culture. The present study revealed that cells treated with *trans*-resveratrol were morphologically more preserved than those in the hypoxic OGD group. Cell viability also increased significantly following the treatment of *trans*-resveratrol as assessed by MTT, NRU, and LDH assays. The present study involves three specific tests to assess the restoration capabilities of *trans*-resveratrol in PC-12 cells. All three assays, namely, MTT, NRU, and LDH release, were found to have equal sensitivity in for *trans*-resveratrol protection. The data reveals that *trans*-resveratrol interacts with the proteins of various cellular organelles as LDH and MTT assays represent membrane depolarization and mitochondrial catabolism, respectively, while the NRU assay is associated with lysosomal bioaccumulation and activity. We previously reported that *trans*-resveratrol protects OGD insulted PC12 cells from apoptosis by down-regulating the apoptotic genes and upregulating the antiapoptotic genes, suggesting that *trans*-resveratrol might exert a neuroprotective effect through an antiapoptotic mechanism.[14] In the present study, treatment of *trans*-resveratrol attenuated the hypoxia induced increased in $[Ca^{2+}]_i$ and reactive oxygen species generation, and increased the expression of superoxide dismutase and catalase in a dose dependent manner, suggesting that the protective effects might be partly associated with its antioxidation function against OGD induced oxidative stress in PC12 cells. The cytoprotective role of *trans*-resveratrol through regulation of cellular homeostasis has been suggested using in vivo and in vitro models.[25,26] Superoxide dismutase and catalase which form the main defense against free radicals have been the focus of experiments pertaining to hypoxic/ischemic injury.[27] It seems that these enzymes are related in such a way that the decrease in the activity of a particular enzyme will also decrease the functioning efficiency of the others.[28]

During hypoxia, translation of the α subunits of HIF is regulated with an influx of extracellular calcium. HIF-1α is not present in normal cells but induced under hypoxic conditions. The HIF-1α subunit is continuously synthesized and degraded under normoxic conditions, while it accumulates rapidly following exposure to low oxygen tensions.[8] Similarly, in the present investigation, OGD given cells showed significant induction in mRNA expression of HIF-1α gene and the treatment of *trans*-resveratrol reduced the expression significantly. Hypoxia promoted an enhanced secretary response of excitable cells via formation of a novel Ca^{2+} influx pathway specifically through L-type Ca^{2+} channels.[12,17] PC12 cells, like many excitable neuronal or neuroendocrine cells, possess multiple types of voltage-gated Ca^{2+} channels such as Cav1.2 and TRPM7 channels.[17,29] In hypoxia, Cav β subunits increase membrane expression of Cav1.2 channels and gating of TRPM7 mediates Ca^{2+} overload which causes damage to neurons. Our results also show significant upregulation of Cav β3 and TRPM7 expression at both the transcription and translation levels during OGD insult in comparison to normoxia. *trans*-Resveratrol inhibited the expression of Cav β3 and TRPM7 significantly down to the level of normoxia in post-treatment and whole-treatment groups. The significant reduction in the expression of Cav β3 and TRPM7 showed the protective potential of *trans*-resveratrol. Aarts et al. used a similar model of hypoxia (OGD) to unveil the activation of a cation conductance (I_{OGD}), which results in Ca^{2+}overload.[29] The discovery of a new gate leading to neuronal death opens an exciting new spectrum of possibilities for therapy of brain hypoxia and potentially other neuropathological conditions.

A rapid activation of heat shock proteins (hsp-27) and STAT pathway provides cells with a vital mechanism for responding to various extracellular stimuli, including ischemic stress.[30−32] Our results also show a rapid activation of hsp-27 and STAT3 at both the mRNA and protein levels during oxygen-glucose deprivation followed by reoxygenation. In vitro studies have demonstrated that STAT3 potentiates antiapoptotic signals through the induction of bcl-2 or bcl-xL genes.[33] These results explore a novel mechanism by which hsp-27 confers neuronal protection and provides an upstream target for neuroprotective intervention in cerebral ischemia.

Under ischemic conditions, energy metabolism fails, and severe reduction in mRNA and protein synthesis occurs in the ischemic core region.[34] The results of the present study provide evidence regarding global changes in gene expression in which HIF-1α, Cav β3, TRPM7, hsp-27, and STAT3 required for adaptation to hypoxia are significantly modulated during hypoxia, probably due to a compensatory response of the cells, which may play a protective role in damaged tissue.

In summary, our data provide a link between several mechanisms thought to cause irreversible neuronal damage in ischemia. We are reporting effective dose and optimal timing of treatment with *trans*-resveratrol to protect OGD-R induced ischemia in PC12 cells. This protection in hypoxic PC12 cells

was achieved by preventing reactive oxygen species generation and intracellular Ca^{2+} level while inhibiting the expression of HIF-1α, Cav β3, TRPM7, hsp-27, and STAT3, thereby increasing the expression of antioxidant defense enzymes. A precise understanding of the interplay of these mechanisms can lead to the development of pharmacological strategies to minimize acute brain damage. The acquired knowledge from this study will provide new insights assisting the development of novel therapeutic strategies to attenuate acute brain damage associated with cerebral ischemia.

METHODS

Cell Culture. PC12 cell line was provided by the National Centre for Cell Sciences (NCCS), Pune, India. Cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 2.5% fetal bovine serum (FBS), 15% horse serum, 0.2% sodium bicarbonate, and antibiotic-antimycotic solution (100×, 1% of medium). Cells were grown at 37 °C in 5% CO_2/95% atmosphere under humid conditions. Medium was changed twice weekly and cultures were split at a ratio of 1:6 once a week. Prior to experiments, cells were assessed for cell viability by trypan blue dye exclusion test, and only cell batches showing more than 95% viability were used in the study.

Reagents and Chemicals. *trans*-Resveratrol and all the specified chemicals, reagents, and diagnostic kits were purchased from Sigma Aldrich unless otherwise stated. Dulbecco's modified Eagle's medium, antibiotics, fetal bovine serum, and horse serum were purchased from Gibco.

Oxygen-Glucose Deprivation (OGD-R). Cells were collected by centrifugation at 600 rpm for 6 min. Following two washes with sterile phosphate-buffered saline (PBS; pH 7.4), cells were resuspended in DMEM having all the standard components except glucose and were allowed to grow at 37 °C in an atmosphere 5% CO_2-/94% N_2/1% O_2 under humid conditions for 6 h. Immediately following the OGD insult for 6 h, fresh medium containing glucose (final concentration 4.5 mg/mL) was added to the cultures. To make conditions identical, glucose free medium used during OGD insult was not aspirated from the cultures as the secretions collecting in the medium during OGD insult might contribute to the detrimental effects of reoxygenation process. Cultures growing in medium were then subjected to reoxygenation for 24 h at 37 °C in 5% CO_2/95% atmosphere under humid conditions.[15]

Treatment Schedule. Biologically safe doses of *trans*-resveratrol were determined prior to all experiments. PC12 cells were exposed to different concentrations (1−1000 μM) of *trans*-resveratrol for 24−96 h and MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was subsequently performed. Five experimental groups were designed: (1) pre-treatment, (2) post-treatment, (3) whole-treatment, (4) OGD control, and (5) normoxia basal control group, as depicted in Figure 1. In the pretreatment group, cells were treated with *trans*-resveratrol for 24 h prior to OGD, followed by 6 h of OGD and 24 h of reoxygenation. In the post-treatment group, cells were treated with *trans*-resveratrol during the 24 h reoxygenation period only. In the whole-treatment group, cells were treated with *trans*-resveratrol for the entire period of experiment, 24 h prior to OGD, during 6 h of OGD, and 24 h of reoxygenation. The OGD control group was not given *trans*-resveratrol treatment, whereas in the normoxia basal control group cells received neither OGD insult nor *trans*-resveratrol treatment. On completion of the reoxygenation period, cells from both experimental and control groups were harvested by centrifugation at 350*g* for 6 min.

Mitochondrial Activity by MTT Assay. MTT assay was performed following the protocols of Kashyap et al.[35] In brief, cells (1×10^4) were seeded in poly-L-lysine precoated 96-well culture plates and allowed to adhere properly for 24 h at 37 °C in a CO_2 incubator (5% CO_2/95% atmosphere under humid conditions). The medium was aspirated and replaced with a glucose-free medium, and cells were exposed to OGD insult for 6 h. Next, cells underwent reoxygenation for 24 h as per treatment schedule. Tetrazolium bromide salt (5 mg/mL of stock in PBS) was added (10 μL/well containing 100 μL of cell suspension) 4 h prior to the completion of reoxygenation period, and the cultures were reincubated further until completion of the specific reoxygenation period. The reaction mixture was carefully taken out, and 200 μL of culture grade dimethyl sulfoxide was added to each well by pipetting up and down several times until the contents became solublilized. After 10 min, absorbance was taken at 550 nm, using a multiwell microplate reader (Synergy HT, BioTek). Untreated sets were run simultaneously under identical conditions, except OGD insult, and served as control.

Neutral Red Uptake (NRU) Assay. Neutral red uptake (NRU) assay was performed as previously published.[36] Briefly, cells (1×10^4) were seeded in poly-L-lysine precoated 96-well culture plates and treated as described for MTT assay. On the completion of respective incubation periods, the test solution was aspirated and cells were washed twice with PBS. Cells were then incubated for 3 h in medium supplemented with neutral red (50 μg/mL). Then the medium was washed off rapidly with a solution containing 0.5% formaldehyde and 1% calcium chloride. Cells were then incubated further for 20 min at 37 °C in a mixture of acetic acid (1%) and ethanol (50%) to extract the dye. Plates were read at 540 nm absorbance using a multiplate reader (Synergy HT, Bio-Tek). The values were compared with control sets, run under identical conditions without the test compound.

Lactate Dehydrogenase (LDH) Release Assay. Lactate dehydrogenase (LDH) release assay is a method to measure the membrane integrity as a function of amount of cytoplasmic LDH released into the medium. This assay was carried out using commercially available LDH assay kit for in vitro cytotoxicity evaluation (TOX-7, Sigma). The assay is based on the reduction of NAD by the action of LDH. Reduced NAD ($NADH^+$) is utilized in the stochiometric conversion of a tetrazolium dye. The resulting colored compound is measured using a multiwell plate reader (Synergy HT, BioTek) at absorbance wavelengths of 490 and 690 nm. In brief, after the ischemic insult and desired reoxygenation period, cells were processed for LDH release assay similar to the MTT assay. Culture plates were removed from the CO_2 incubator as per the experimental schedule and centrifuged at 250*g* for 4 min. The supernatant of each well was transferred to a fresh flat bottom 96-well culture plate and processed further for enzymatic analysis as per the manufacturer's instructions.

Intracellular Calcium Levels $[Ca^{2+}]_i$. Alterations in the levels of intracellular calcium in cells of the experimental group versus control were assayed by fluorometric quantification of hydrolyzed acetylmethyl ester of quinoline tetracarboxylic acid (Quin-2AM).[37] Cells (1×10^6) were incubated in 3.0 mL of assay buffer (120 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 5 mM $NaHCO_3$, 6 mM glucose, 6 mM $CaCl_2$, 25 mM Hepes, pH 7.4) along with Quin-2AM (25 μM final concentration) at 37 °C for 45 min in the dark. The assay mixtures were then centrifuged at 1000 rpm for 10 min, and the pellets were washed three times to remove excess Quin-2AM. Finally, pellets were suspended in 1.0 mL of assay buffer. Fluorescence "*R*" was measured on a spectrofluorimeter (LS 55 Perkin-Elmer, U.K.) using an excitation and emission wavelengths of 330 and 492 nm, respectively. Fluorescence "*R* minimum" (R_{min}) was observed after the addition of 0.1% sodium dodecyl sulfate (SDS) and EGTA (10 mM). The fluorescence "*R* maximum" (R_{max}) was determined after adding 7 mM $CaCl_2$. Intracellular calcium levels were then calculated using the formula: Intracellular $[Ca^{2+}] = K_d(R - R_{min})/(R_{max} - R)$, where K_d is the dissociation constant of the Quin-2AM−Ca^{2+} complex and is equivalent to 115 nm.

Fluorescent Microscopy of Reactive Oxygen Species (ROS). Hypoxia induced reactive oxygen species generation was assessed in PC12 cells using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma Aldrich) dye as the fluorescence agent. Nonspecific esterases are known to cleave the acetoxymethyl group of DCFH-DA, resulting in formation of a nonfluorescent charged molecule that cannot cross the cell membrane. The intracellular reactive oxygen species oxidizes these charged nonfluorescent molecules to dichloro-

Table 1. Oligonucleotide Primer Sequences Used in Real-Time PCR[q] Using SYBR Green Dye

gene name	forward primer (5′-3′)	reverse primer (5′-3′)
HIF-1α	GTTTACTAAAGGACAAGTCACC	TTCTGTTTGTTGAAGGGAG
Cav β3	GGCACTTCTGTCCCATCAGC	CACCCGCACTGCTCCTGT
TRPM7	GCCGCCCTCTTCCTTTCT	ATTTGCTCCTCTGTCCACATTTG
STAT3	TCCTGGTATCCCCACTGGTC	TTCCGAATGCCTCCTCCTT
hsp-27	ATCACTGGCAAGCACGAAGA	GGGTGAAGCACCGAGAGAT
HPRT	CCAGTCAACGGGCGATATAA	CTTGACCAAGGAAAGCAAGG

fluorescein (DCF) fluorescent product. The fluorescence measurement was done by intracellular quantification using a fluorescence microscope. The assay was carried out following the protocol of Kashyap et al., 2011.[38] In brief, cells (5×10^4 per well) were seeded in a poly-L-lysine precoated tissue culture chamber slide and allowed to adhere. The experimental setup for reactive oxygen species generation was similar to other end points. On the completion of the reoxygenation period of 24 h, cells were washed twice with PBS and incubated for 30 min in the dark in incomplete culture medium containing DCFH-DA (20 μM). After 30 min, slides were washed twice with PBS and mounted for microscopic analysis. Intracellular fluorescence was measured using an upright fluorescence microscope by capturing the images (Nikon Eclipse 80i equipped with Nikon DS-Ri1 12.7 megapixel camera). Quantification of fluorescence was done using image analysis software (Leica Q-win 500), and data expressed in fold of unexposed control.

Superoxide Dismutase (SOD) Activity. The activity was measured using a commercially available kit for superoxide dismutase activity (catalog no. 706002; Cayman Chemicals) following the protocol provide by manufacturer. After completion of the reoxygenation period, cells were collected by centrifugation at 1000*g* for 10 min at 4 °C and sonicated in Hepes buffer (20 mM, pH 7.2, containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose). The cell suspension was then again centrifuged at 1500*g* for 5 min at 4 °C, and the supernatant was collected for assay. The assay sample (10 μL) was mixed with 200 μL of radical detector (supplied in the kit). Reaction was initiated by adding 20 μL of xanthine oxidase. The plate was incubated on a shaker for 20 min at room temperature, and the absorbance was read at 450 nm using a microplate reader (Synergy HT, BioTek). A standard curve was plotted using the SOD standard supplied in the kit.

Catalase Activity. Activity was measured using a commercially available kit for catalase activity (catalog no. 707002; Cayman Chemicals) following the protocol provided by manufacturer. Following the completion of the reoxygenation period, cells were collected by centrifugation at 1000*g* for 10 min at 4 °C, sonicated in 1.0 mL of cold buffer (50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA), and centrifuged at 10 000*g* for 15 min at 4 °C. The supernatant was collected for assay. Then 100 μL of assay buffer (supplied in the kit), 30 μL of methanol, and 20 μL of sample were mixed in the 96-well plate. Reaction was initiated by adding 20 μL of hydrogen peroxide (0.882 M) and incubating in a shaker for 20 min at room temperature and then stopped by adding 30 μL of potassium hydroxide. Chromogen (30 μL) was added and incubated for 10 min followed by the addition of potassium periodate (10 μL). Plates were kept at room temperature for 5 min and read at 540 nm using a microplate reader (Synergy HT, BioTek). Bovine liver catalase (supplied in the kit) was used as a positive control in the assay. A standard curve was plotted using formaldehyde between 0 and 75 μM.

Transcriptional Profiling of Hypoxia Regulating Genes. *RNA Isolation.* Total RNA was isolated using Trizol reagent (Gibco, BRL) as per the manufacturer's protocol and checked for purity and yield using a Nanodrop ND-1000 spectrophotometer V3.3 (Nanodrop Technologies Inc.). The quality of RNA was also assessed by running it on a 2% denaturing agarose gel. Total RNA (1 μg) was reverse-transcribed into cDNA by using a Super Script III first strand cDNA synthesis kit (catalog no. 18080-051, Invitrogen Life Science) using random hexamer primers.

Real Time PCR (qRT-PCR). Quantitative real time PCR was performed using an ABI 7900HT sequence detection system (Applied Biosystems). Real time reactions were carried out with a Power SYBR Green PCR kit (M/s Applied Biosystems) as per the protocol provided by the manufacturer. For PCR amplification, an initial denaturation was performed at 95 °C for 15 min followed by 40 cycles of denaturation (95 °C for 15s), annealing, and extension (60 °C for 60s). All reactions were performed in triplicate for each sample. A dissociation reaction was also carried out for each primer set to confirm that only one product was formed (list of primers in Table 1). Further, samples were electrophoresed in a 2.0% agarose gel to ensure gene specific amplification. The specificity of each primer was analyzed by melting curve and no template controls (NTCs) for respective primers. HPRT was used as an internal control to normalize the data. OGD-reoxygenation induced alterations in mRNA expression were expressed in relative quantity (RQ) comparing respective normoxia controls. The restoration in the OGD induced damage was calculated by comparing the data of OGD induced changes with *trans*-resveratrol treated groups.

Western Blot Analysis. Western blot analysis for the expression of HIF-1α, Cav β3, TRPM7, STAT3, hsp-27, and HPRT was carried out following the standard protocol described earlier by our group.[38] In brief, cells from respective groups were pelleted and lysed using CelLytic M cell lysis reagent (catalog no. C2978, Sigma) in the presence of a protein inhibitor cocktail (catalog no. P8340, Sigma). After protein estimation by using a BCA protein assay kit (catalog no. G1002, Lamda Biotech, Inc.), equal amounts of proteins were loaded in a gel, electrophoresed, and then transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking for 2 h at 37 °C, membranes were incubated overnight at 4 °C with anti-protein primary antibodies specific for HIF-1α, STAT3, hsp-27, and Cav β3 (1:500, Santa Cruz); TRPM7 (1:500, Millipore); and HPRT (1:1000, Abcam) in blocking buffer (pH 7.5). The membranes were then incubated for 2 h at room temperature with secondary anti-primary IgG conjugated with horseradish peroxidase (Calbiochem). Blots were developed using either luminol (Thermo Scientific) or TMB-H_2O_2 (Sigma), and densitometry for protein specific bands was done with a Gel Documentation System (Alpha Innotech) with the help of Alpha Ease FC Stand Alone V. 4.0.0 software.

Statistical Analysis. Results are expressed as mean ± SEM of three experiments each carried out in triplicate. Statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's posthoc test to compare the inter- and intragroup variations with PRISM software. The values depicting $P < 0.05$ were considered as statistically significant.

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M.A., V.K., and A.B.P. conceived and designed the experiments. M.A., V.K., A.K.S., M.P.K., V.K.K., M.A.S., and A.B.P. performed the experiments. M.A., V.K., and A.B.P. analyzed the data. M.A., V.K., and A.B.P. wrote the paper.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

OGD, oxygen-glucose deprivation; R, reoxygenation; ROS, reactive oxygen species; HIF-1α, hypoxia induced factor-1 alpha; Cav β3, Cav-beta 3; STAT3, signal transducer and activator of transcription 3; hsp-27, heat shock protein 27; TRPM7, transient receptor potential melastatin 7; VEGF, vascular endothelial growth factor

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