Akt activates NOS3 and separately restores barrier integrity in H2O2-stressed human cardiac microvascular endothelium

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MICROCIRCULATORY DYSFUNCTION is an important etiological component of ischemia-reperfusion injury. A significant percentage of victims of cardiac arrest, despite being initially resuscitated, die of heart-related causes within hours due to recurrent arrhythmias and cardiovascular collapse (4, 23, 28, 47). Recent work suggests that these cardiac arrest events are temporally associated with interruptions in microvascular blood flow to the heart that begin within minutes of cardiopulmonary resuscitation (CPR) and may affect the return of spontaneous circulation (ROSC) (17). Furthermore, serious microvascular dysfunction in critical organs such as the heart and brain is seen within hours of ROSC and is associated with critical organ dysfunction (1, 8).

The mechanism of microvascular endothelial dysfunction in the post-cardiac arrest heart is not well known. Our studies in a swine model of cardiac arrest suggest that oxidant stress increases significantly within minutes of CPR and peaks 5–10 min after ROSC (20). Oxidant stress caused by a surge in reactive oxygen species (ROS) generation during reoxygenation (54) can disrupt microvascular integrity and interrupt blood flow (25, 38, 56). Although responses to ROS in several endothelial cell types have been reported, how human cardiac microvascular endothelial cells (HCMVEC) respond to ROS has not been well characterized. Other endothelial cell types lose monolayer integrity when exposed to hydrogen peroxide (H2O2) with variable ability to recover over time (9, 24, 25, 31, 36, 44, 51).

HCMVEC response to ROS could impact heart function via changes in endothelial barrier function that then disrupt tissue blood flow. In addition, these cells serve as an important source of nitric oxide (NO), which regulates blood flow (22, 27, 40). This NO could also affect surrounding cardiomyocyte function. Our work in heart cell models of ischemia-reperfusion injury suggests that NO generation, possibly via survival kinase signaling, can play a highly cardioprotective role (21, 29, 45). In endothelial cells, the survival kinase Akt has been recognized for some time as an upstream regulator of such cardioprotective NO generation (18), but only recently has it been implicated as a positive regulator of endothelial chemotaxis and barrier integrity (31).

Given that ROS can activate Akt (11, 39), and Akt is upstream of both cardioprotective NO signaling and tight junction regulation, we tested the ability of H2O2 to affect HCMVEC monolayer integrity via Akt and NO synthase (NOS)3 signaling. Here we show that H2O2-induced NOS3 activation is, in part, phosphatidylinositol 3-kinase (PI3K)-Akt dependent. We also show that Akt is critical for the restoration of H2O2-impaired HCMVEC monolayer integrity apart from NO signaling. Our findings support the notion that Akt-dependent signaling is pivotal for the functional protection of the heart during conditions of increased oxidant stress.

**MATERIALS AND METHODS**

Materials. Cell culture reagents including complete microvascular endothelial cell growth medium-2 (EGM-2MV) and phenol red-free endothelial basal medium-2 (EBM-2) were purchased from Lonza...

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(Walkersville, MD). The selective Akt inhibitor Akt/PKB signaling inhibitor-2 (API-2) was obtained from EMD Chemicals (Gibbstown, NJ). Propidium iodide (PI), Nω-nitro-L-arginine methyl ester (L-NAME), H2O2, LY-294002, and β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). 4,5-Diaminofluorescein diacetate (DAF-2 DA) was from EMD Biosciences (San Diego, CA). The monoclonal antibody for endothelial NOS (eNOS; NOS3) was purchased from BD Transduction Laboratories (BD Biosciences, Franklin Lakes, NJ). Vascular endothelial (VE)-cadherin polyclonal antibodies were purchased from Cayman Chemical (Ann Arbor, MI), and zona occludens-1 (ZO-1) polyclonal antibodies were purchased from Invitrogen (Carlsbad, CA). All other antibodies and Akt kinase assay kit were from Cell Signaling Technology (Beverly, MA). The ApoAlert caspase-3 assay plate was obtained from Clontech (Mountain View, CA).

**Cell culture.** HCMVEC were purchased from Lonza and cultured in complete EGM-2MV medium containing 5% FBS. Passages between 5 and 8 were used for all experiments. Cells were grown at 37°C in a humidified atmosphere containing 5% CO2. For all signaling and transendothelial electrical resistance measurement experiments, cells were starved for 1 h in serum-reduced EBM-2 medium containing either 0.02% FBS or 0.1% BSA and then treated in the same medium.

**Perfusion system and media composition.** HCMVEC grown on 25-mm glass coverslips were placed inside a Sykes-Moore chamber (1.2 ml; Bellco Glass, Vineland, NJ) as described previously (53). The chamber and inflow tubing were maintained at 37°C. Phenol red-free EBM-2 medium with 0.02% FBS was prebubbled with 5% CO2, 21% O2, and 74% N2 gas for 30 min before each experiment. The medium was continuously channeled into the chamber at a flow rate of 0.25 ml/min. Oxygen tension (PO2), pH, and the temperature of the perfusate were controlled as previously described (23, 25). Tubing supplying medium to the chamber was made of stainless steel and PharMed polymer (Cole-Parmer Instrument, Chicago, IL).

**Video/fluorescent microscopy.** A Nikon TE 2000-U inverted phase/epifluorescent microscope was used for cell imaging. Phase contrast Hoffman modulation optics and a charge-coupled device (CCD) camera were used to monitor morphologic changes over time in the...
same field of cells (~70 × 90 μm). Fluorescent images were acquired from a cooled Hamamatsu slow-scanning PC-controlled camera (CoolSnap; Photometrics), and changes in fluorescent intensity were quantified with MetaMorph software (Universal Imaging, Downingtown, PA).

Cell viability. Cell viability was assessed with the fluorescent exclusion dye PI (5 μM) with excitation of 540 nm and emission of 590 nm. PI fluorescence was used to quantify cell death throughout the entire experiment in a selected field of endothelial cells. At the end of the experiment, all cells were permeabilized with digitonin (100 μM). Cell death was expressed as the PI fluorescence at any given time point relative to the maximal fluorescence observed after cell permeabilization with digitonin.

Measurement of intracellular NO production. NO generation by HCMVEC was evaluated in the perfusion system by monitoring fluorescence of the NO-selective probe DAF-2 DA (1 μM) following cell treatment with 500 μM H$_2$O$_2$ for 10 min. Images were taken at 30 min following H$_2$O$_2$ washout with excitation of 480 nm and emission of 520 nm. Fluorescence intensities of cells were measured using MetaMorph software and expressed as arbitrary units.

Measurement of HCMVEC transendothelial electrical resistance. HCMVEC were grown to confluence in polycarbonate wells containing gold microelectrodes, and transendothelial cell electrical resistance (TEER) measurements were performed using an electrical cell substrate impedance sensing system (Applied Biophysics, Troy, NY) as described previously (27). These measurements provide a highly sensitive biophysical assay that indicates the state of cell shape and focal adhesion (19). TEER values from each microelectrode were pooled at discrete time points and plotted versus time as means ± SE. Each experiment was initiated with HCMVEC monolayer resistance of no less than 1,200 ohm. H$_2$O$_2$ (100, 250, or 500 μM, as indicated) was present all course during these experiments.

Caspase activity assay. HCMVEC treated with or without H$_2$O$_2$ were harvested at selected times, washed twice with cold phosphate-buffered saline (PBS), lysed, and stored at −80°C until analysis. Cells stimulated with 10 μM staurosporine for 3 h were used as a positive control. Caspase-3 activity was measured by the ApoAlert caspase profiling assay, as described previously (41).

Western blotting. After H$_2$O$_2$ or control treatments, HCMVEC were washed twice with cold PBS and incubated in lysis buffer (Cell Signaling, Danvers, MA) on ice for 5 min followed by brief sonication. Protein concentrations were determined with a Bradford protein assay. Proteins (50 μg) were separated on 7.5% or 10% gels by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblotting with appropriate primary antibody (1:1,000 dilution) overnight at 4°C. After washing and incubation with appropriate secondary antibody, bands were detected with enhanced chemiluminescence kit (Amersham Pharmacia). Densitometric analysis of Western blots was carried out using Quantity One Software (Bio-Rad, Richmond, CA).

Akt activity assay. The activity of Akt was assessed using a nonradioactive Akt kinase assay kit (Cell Signaling), according to the manufacturer’s instructions. Briefly, immunoprecipitation of endogenous Akt was performed overnight on 250 μg of endothelial cell extract with immobilized anti-Akt antibody at 4°C. Following precipitation, the immunoprecipitate was centrifuged, washed twice with lysis and kinase buffers, and then incubated with 1 μg of GSK fusion protein and ATP for 30 min at 30°C in a final volume of 50 μl. The reaction was stopped by adding loading buffer, and the proteins were separated on 10% gels by SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phospho-GSK3α/β Ser-21/9 antibody. Western blots were developed with the enhanced chemiluminescence kit (Amersham Pharmacia).

Statistical analysis. Treatment and control groups were used in sets containing cells cultured on the same day to eliminate variability due to cell batch. Each experiment was performed at least three times. Data are presented as means ± SE, and two-tailed unpaired t-tests were performed as post hoc tests of significance, with P < 0.05 considered to be significant.

RESULTS

High concentrations of H$_2$O$_2$ are required to activate Akt in HCMVEC. H$_2$O$_2$ is known to activate NOS3 in multiple endothelial cell types via Akt signaling pathways (34, 48, 49). To test whether H$_2$O$_2$ can activate Akt in HCMVEC, we used increasing concentrations of H$_2$O$_2$ in an attempt to induce the phosphorylation of Akt at major regulatory sites including Ser-473 and Thr-308 (10, 12, 18). We found that high micromolar concentrations of H$_2$O$_2$ were needed to stimulate Akt phosphorylation at both sites. A detectable increase in phosphorylation occurred after 250 μM H$_2$O$_2$ with substantially more phosphorylation seen after exposure to a 500 μM H$_2$O$_2$ concentration for 10 min (Fig. 1, A and B). Such levels of H$_2$O$_2$ are reasonable for modeling HCMVEC exposure to oxidant stress in the heart. For example, concentrations of 80–480 μM of H$_2$O$_2$ can be electrochemically detected from stimulated leukocytes at normal plasma concentrations (32).

### Fig. 2. H$_2$O$_2$ does not induce cell death in HCMVEC. A: HCMVEC were treated with 500 μM H$_2$O$_2$ for 30 min and then with basal medium for 2.5 h or with 10 μM staurosporine for 3 h, and caspase-3 activity was assessed in cell lysates using caspase-3-selective fluorogenic substrate (means ± SE; n = 3). B: HCMVEC were treated with 500 μM H$_2$O$_2$, and cell death was monitored by propidium iodide (PI) exclusion. Data are presented as means ± SE of 6 independent experiments. *P < 0.05; ***P < 0.001.
We further studied the time dependency of Akt Ser-473 and Thr-308 phosphorylation with 500 μM H₂O₂. Akt phosphorylation occurred at both sites by 1.5 min, reached a maximum at 10 min, and sustained this level of phosphorylation throughout 30 min of H₂O₂ stimulation (Fig. 1, C and D). However, Akt activity, as measured by phosphorylation of GSK fusion protein, peaked at 10 min and was only half of the maximum by 30 min of stimulation (Fig. 1E). Given that significant activation occurred by 10 min H₂O₂ exposure, we also tested whether the removal of H₂O₂ for 20 min (versus prolonged stimulation for 30 min) had different effects. Despite the removal of H₂O₂ for 20 min after the initial 10-min stimulation, a significant level of Akt phosphorylation and Akt activity at 30 min was maintained. This level of activation was not significantly different from the level observed with continuous 30-min H₂O₂ exposure (Fig. 1, C–E).

High concentrations of H₂O₂ do not induce HCMVEC caspase-3 activation or cell death. To evaluate whether 500 μM H₂O₂ induced cell death in HCMVEC, we measured caspase-3 activation and cell viability following 30 min H₂O₂ exposure. We found that 500 μM H₂O₂ does not increase but rather decreases the background level of caspase-3 activity in HCMVEC (Fig. 2A). Cell death, as determined by PI exclusion, did not exceed 5% during 30 min of exposure to 500 μM H₂O₂ or during the subsequent 60 min in replacement medium (Fig. 2B). Taken together, these data indicate that the concentrations of H₂O₂ used did not increase cell death in HCMVEC.

H₂O₂-induced activation of NOS3 via Ser-1177 phosphorylation and Thr-495 dephosphorylation. Maximal activation of eNOS (NOS3) requires phosphorylation at Ser-1177, which is downstream of Akt signaling, and concomitant dephosphorylation of Thr-495 via an Akt-independent process (34, 48, 49). As shown in Fig. 3, A and B, H₂O₂ induces maximal NOS3 phosphorylation in HCMVEC at Ser-1177 and dephosphorylation at Thr-495 sites by 1.5 min after treatment. The phosphorylation-to-dephosphorylation ratio between these two sites peaked at 1.5 min relative to the ratio seen at 10 min after H₂O₂ cell stimulation (Fig. 3C). We also measured H₂O₂-induced NO production using the NO-specific fluorescent probe DAF-2 DA. As shown in Fig. 4, NO generation was substantially upregulated by H₂O₂ and blocked by the NOS inhibitor L-NAME. The selective Akt inhibitor API-2 also attenuated H₂O₂-induced NO production. These results implicated Akt as a regulator of NOS3 in ROS-stressed HCMVEC. To further confirm the involvement of the PI3K-Akt pathway in the regulation of NOS3, we tested the effect of the PI3K inhibitor LY-294002 and the Akt inhibitor API-2 on H₂O₂-induced Akt phosphorylation. Both inhibitors blocked Akt phosphorylation at its Ser-473 and Thr-308 sites (Fig. 5, A and B). In addition, both PI3K and Akt inhibitors blocked H₂O₂-induced NOS3 phosphorylation at Ser-1177 and Thr-495 sites (Fig. 5, A and B).
phosphorylation at Ser-1177 (Fig. 5C), the site regulated by Akt (13, 33). PI3K and Akt inhibitors, however, did not significantly affect the Thr-495 dephosphorylation of NOS3 caused by H2O2 (Fig. 5D). This confirms that, as in other endothelial cell types, the PI3K-Akt pathway is not involved in the regulation of the NOS3 Thr-495 site. This site has been found to be phosphorylated by protein kinase C and dephosphorylated by protein phosphatase 1 and 2A and calcineurin (5, 15, 16).

Akt is critical for restoration of H2O2-induced barrier permeability. Oxidative stress in other endothelial cell types results in the loss of tight junction function and increased barrier permeability (24–26, 42, 50, 51). Rat lung microvascular endothelial cells quickly restore monolayer integrity within 10–15 min of treatment with a low concentration of H2O2 (100 μM) (42), whereas bovine pulmonary artery endothelial cells fail to do so even several hours after initial H2O2 exposure (50). We tested whether H2O2 exposure causes a similar loss of monolayer integrity in HCMVEC. As measured by TEER in HCMVEC, we found that H2O2 quickly disrupted monolayer integrity in a dose-response fashion from 100 to 500 μM concentrations (Fig. 6A). HCMVEC began to restore TEER within 30–60 min, reaching baseline TEER levels within hours, despite continuous exposure to H2O2 concentrations of up to 500 μM. Consistent with this, we found that H2O2 exposure was associated with loss of both VE-cadherin-mediated adherence and ZO-1-mediated tight junctions (Fig. 7). Complementary to the TEER data, both VE-cadherin- and ZO-1-mediated junctions were restored in HCMVEC 3 h after stimulation with 250 μM H2O2 (Fig. 7). Given that Akt and NOS3 are activated within 1.5–30 min after H2O2 exposure, we further tested whether they regulate these changes in HCMVEC barrier integrity. As seen in Fig. 6B, the Akt inhibitor API-2 (10 μM), effective in blocking Akt and NOS activation during ROS exposure, blocked the restoration of endothelial cell TEER. By itself, API-2 had no effect during baseline conditions on the integrity of cell-cell interactions. On the contrary, NOS inhibition with L-NAME at a concentration previously shown to inhibit NO generation (200 μM) had no effect on either baseline TEER, early loss of monolayer integrity, or its subsequent recovery after H2O2 exposure (Fig. 6C). These data suggest that Akt plays an important
DISCUSSION

Ischemia-reperfusion of cardiac tissue in vivo and cardiomyocytes in vitro results in a massive generation of different forms of ROS including H$_2$O$_2$ (2, 52, 54). This heart cell ROS peaks within 10 min of reperfusion (52). Similarly, even minutes of hypoxia, as occurs during cardiac arrest, can initiate cardiomyocyte ROS generation and release by mitochondria that initiate important stress responses (30, 53). In addition to exposure to neighboring heart cell ROS, endothelial cells are exposed to oxidants from the vascular space. Leukocyte activation occurs early during cardiac arrest and can generate H$_2$O$_2$ in concentrations reaching 500 μM (6, 32). Consistent with this, we have reported in a swine model of cardiac arrest that serum and tissue isoprostane levels increase almost three-fold within 10 min after ROSC (20). Such rapid ROS generation during minutes of reoxygenation could disrupt microvascular integrity and interrupt blood flow (25, 38, 56) and is likely related to the microvascular dysfunction that appears within the heart minutes after resuscitation (17). In the present study, the substantial loss of HCMVEC barrier integrity after oxidant stress is also consistent with the microvascular dysfunction seen in critical organs such as the heart and brain within hours of resuscitation (1, 8, 55).

This study was conducted to better understand important oxidant stress responses within a newly available endothelial cell type: the HCMVEC. Different endothelial cell types appear to respond uniquely to ROS, and understanding how the heart responds to the oxidant stress of cardiac arrest is important if we are to develop better treatments for a disease with 95% mortality (35, 47). Despite initially successful resuscitation in many cardiac arrest patients, a significant number die within minutes to hours from heart-related complications, including severe cardiovascular dysfunction and hemodynamic collapse (28, 43). In addition, there are no treatments currently given during CPR designed to specifically modulate oxidant stress or restore microvascular function. This study builds on prior work by focusing on heart-specific microvascular endothelial cells. Our finding that HCMVEC restore barrier function within hours of significant oxidant stress stands in contrast with other endothelial cell types, which are unable to restore barrier integrity after similar intensity of oxidant stress and within the same time frame (50, 51). In the setting of cardiac arrest and increased cardiovascular oxidant stress during CPR and after ROSC, the timing and extent of HCMVEC barrier function failure could impact the early cardiovascular collapse seen in many post-cardiac arrest patients (47). The current work suggests that Akt may be an important modulator of barrier integrity restoration in the heart during conditions of oxidant stress.

ROS and endothelial cell barrier integrity. The effect of H$_2$O$_2$ on vascular permeability and signaling in endothelial cells of different origin in vitro has been studied, but HCMVEC have only recently become available. In other cell types it is known that H$_2$O$_2$ can cause endothelial cell monolayer permeability due to the disruption of cell tight junctions (9, 31, 44) with ERK (14, 24) and p38 (25, 36, 51) MAP kinases mediating these effects of H$_2$O$_2$. The current data suggest that...
HCMVEC adapt after oxidant stress and restore TEER. In our experiments, HCMVEC could restore TEER within 30 min after treatment with 100 μM H₂O₂ and within 3 h after stimulation with 500 μM H₂O₂. Importantly, HCMVEC restored both adherence (VE-cadherin based) and tight (ZO-1 based) junctions, confirming a strong adaptive potential of these cells (Fig. 7). Similar to HCMVEC, rat lung microvascular endothelial cells could restore TEER within 30 min after stimulation with 100 μM H₂O₂ (26). In contrast, bovine pulmonary artery endothelial cells (50) and bovine lung microvascular cells (51) do not restore monolayer integrity for several hours after exposure to as low as 100 μM H₂O₂. Collectively, this indicates relatively high adaptive potential of HCMVEC during exposure to relatively high oxidant stress.

Fig. 7. Dynamic changes in the adherence and tight junctions in HCMVEC upon treatment with H₂O₂. HCMVEC grown on coverslips were treated with 250 μM H₂O₂ for 20 min or 3 h, and adherence junction [vascular endothelial (VE)-cadherin] and tight junction [zona occludens-1 (ZO-1)] proteins were identified by immunostaining. Note that both VE-cadherin and ZO-1 peripheral continuous localization is disrupted by H₂O₂ treatment (residual contacts between cells 20 min after the treatment are marked by arrows) and then restored back to near normal appearance. Scale bar, 5 μm.
cell, and NO appears to play some role in both permeability and restoration of barrier integrity in some cell types (34, 48, 49). A direct role for NOS3 in preserving the normal structure of endothelial cell junctions in multiple vascular beds, including pulmonary vasculature, was shown using electron microscopy in eNOS−/− mice (40). Conversely, NO has been demonstrated to be involved in ischemia-reperfusion, platelet-activating factor, histamine, and VEGF-induced microvascular permeability (7, 37). However, despite evidence of NOS3 activation and NO generation, NO in this HCMVEC model appeared to play no major role in either the initial permeability or recovery phases. In HCMVEC, NOS3 seems not to be involved in the regulation of H2O2-induced permeability. l-NAME, at concentrations that inhibit NO production (Fig. 4), failed to affect the H2O2-elicted changes in TEER (see Fig. 6D). It is likely that the NO release seen in these cells could play other important roles in the heart. The release of NO by endothelial cells results in vasorelaxation and in overall improvement of circulation through the effect of NO on vascular wall smooth muscle cells (3) and NO modulation of cardiomyocyte contractility (22). In our own heart cell models, increased cardioprotective NO generated early during reperfusion can modulate ROS generation by the mitochondria and attenuate cardiomyocyte injury (29, 45). In addition, exogenous NO (as could be provided by neighboring endothelial cells) is highly protective against ischemia-reperfusion injury (21). Thus the NO generation seen in HCMVEC could serve many cardioprotective roles apart from the regulation of microvascular barrier integrity.

Role of Akt in endothelial cell integrity. The present study examined the role of Akt because it has recently been implicated in endothelial cell barrier function regulation (31, 44). Since it can be simultaneously activated by levels of ROS that activate p38 (39) and induce barrier disruption (51), it is reasonable to think that Akt plays a role in regulating barrier dysfunction in the setting of oxidant stress. In addition, Akt is upstream of NOS3 activation (13) and cardioprotective NO generation (18, 22). Others have shown that H2O2 can activate PI3K-Akt signaling pathways and activate NOS3 via downstream NOS3 Ser-1177 phosphorylation (9, 34, 48). Our results suggesting that 250–500 μM H2O2 activates PI3K-Akt signaling in HCMVEC (see Fig.1) are consistent with this previously reported work. The maximal stimulation of NOS3 Ser-1177 phosphorylation in porcine artery endothelial cells was achieved by 500 μM H2O2 (50). In other reports, NOS3 phosphorylation in the same cell type was achieved by 100 μM H2O2 but not with 500 μM H2O2 (49). In both reports, Akt was shown to be critical for eNOS Ser-1177 phosphorylation. Our finding that ROS-mediated Akt Ser-473 and Thr-308 phosphorylation in HCMVEC is mediated by the upstream activation of PI3K (see Fig.5) is also consistent with work by others using different cell types (9, 49).

In human umbilical vein endothelial cells, Akt has recently been described as a regulator of endothelial chemotaxis that can increase barrier integrity (31). Other work in human pulmonary artery endothelial cells suggests that Akt can improve barrier integrity by phosphorylating the barrier-promoting sphingosine-1-phosphate receptor 1, inducing downstream RhoA/Rac1 signaling to the cytoskeleton (46). Conversely, Akt may mediate ROS stress-induced permeability changes in rat brain endothelial cells (44). In contrast to the current work, this study exposed cells to prolonged superoxide generation using xanthine/xanthine oxidase exposure. Our own results suggest for the first time that Akt in HCMVEC helps restore H2O2-induced permeability, given that the Akt inhibitor API-2 completely blocked HCMVEC ability to regain monolayer integrity (see Fig. 6B). Interestingly, API-2 had no effect on the basal TEER, indicating that Akt is not required to maintain the integrity of established cell contacts.

Conclusion

Our study suggests that H2O2-induced oxidant stress in HCMVEC activates NOS3 via Akt. NOS3/NO are not involved in the regulation of H2O2-affected barrier function in HCMVEC. Akt, independent of NOS3 regulation, proves to be critical for the restoration of barrier integrity in HCMVEC.

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REFERENCES

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