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The Peripheral Benzodiazepine Receptor Modulates Ca^{2+} Transport through the VDAC in Rat Heart Mitochondria

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The voltage-dependent anion channel (VDAC) is a key mitochondrial protein involved in the transport of calcium. Its function is, in part, regulated by associated proteins such as the 18 kD peripheral benzodiazepine receptor (PBR). We tested the hypothesis that an endogenous ligand of the PBR, hemin, could modulate calcium transport by modifying VDAC conductance. In isolated rat cardiac mitochondria, hemin (0–10 μM) exhibited multiple, time-dependent effects. Initially, hemin reduced the calcium uptake rate in a dose-dependent manner, an effect independent of the mitochondrial permeability transition (MPT) as it was not altered by cyclosporine A (CsA). However, subsequent to this inhibitory effect on calcium influx, hemin facilitated MPT as evidenced by greater calcium release following calcium loading. An inhibitory effect of hemin on VDAC conductance was supported by lipid bilayer experiments in which hemin induced channel closure of the VDAC-PBR-ANT complex. These findings indicate that the PBR ligand hemin has complex effects on mitochondrial calcium uptake, consistent with a PBR-dependent effect on VDAC conductance. ***J Clin Basic Cardiol 2008; 11 (online): 24–9.***

Key words: *benzodiazepine receptor, mitochondria, voltage dependent anion channel, VDAC, translocator protein, calcium, permeability transition, MPT, bilayer*

The transport of metabolites and ions across the outer mitochondria membrane is, in part, governed by the voltage-dependent anion channel (VDAC), or mitochondrial "porin" [1, 2]. As such, this 32 kDa protein can play an essential role in regulating the entry of calcium into mitochondria under conditions of increased energy demand or, potentially, under conditions of cytosolic calcium overload as seen in ischemia/reperfusion [3]. In addition to its role in ion and metabolite entry, VDAC may also be a key element in the mitochondria permeability transition (MPT) and apoptosis [4]. These dual functions of VDAC are explained by its relationship to companion proteins such as the adenine nucleotide translocator (ANT), hexokinase, cyclophillin and the 18 kDa peripheral benzodiazepine receptor (PBR) [1] which can result in either a regulatory entry pathway or a mechanism for cell death [5].

The association of VDAC with PBR is extremely close, with some investigators suggesting that the "mitochondrial benzodiazepine receptor" should be considered as the complex of VDAC, the 18 kDa "translocator protein" and the ANT [6]. Regardless of terminology, it is likely that the 18 kDa PBR has an important regulatory role in VDAC function, both in inward ion transport and in permeability transition.

Mitochondrial Ca^{2+} overload is a critical event in myocyte necrotic and apoptotic cell death following ischemia and reperfusion [7]. Since pharmacologic interventions which reduce mitochondrial calcium overload have been shown to protect ischemic myocardium [8, 9], it is possible that modulation of mitochondrial calcium entry through VDAC could be a potential pharmacologic target to limit ischemia/reperfusion injury.

In order to elucidate the regulatory function of the 18 kDa PBR on VDAC-modulated calcium entry into mitochondria, we employed several measures of VDAC and PBR function in isolated mitochondria and lipid bilayers. The experiments tested the following hypotheses: first, the PBR ligand hemin (protoprophyrin IX) limits calcium entry into mitochondria in a dose-dependent manner; second, the PBR ligand hemin modulates permeability transition in the same dose range, and third, the effect of hemin is a consequence of a direct modulatory effect of PBR on VDAC.

Materials and Methods

Animals

Male Sprague-Dawley rats, 250–300 gr, were used for all the experiments and were obtained from Charles River (Boston, MA). The rats were housed and maintained following the protocols approved by the Institutional Animal Care and Use Committee (IACUC), University of California, Davis. Experiments were conducted as approved by IACUC and in accordance with the animal care guidelines set by the National Institutes of Health.

Mitochondrial Isolation

Rat heart mitochondria were isolated based on standard techniques used in our laboratory [10, 11]. Briefly, the left ventricle was dissected out and quickly washed three times in cold (4 °C) isolation buffer (IB; 250 mM Sucrose, 5 mM Tris-HCl, 1 mM EDTA; pH 7.4). The tissues were then processed in a PowerGen 1800D homogenizer (Fisher Scientific, Pittsburg, PA), twice for 10 sec each with a one-minute interval. To further extract the heart mitochondria, a series of differential centrifugation was performed in a Sorvall RC-2B at 4 °C. The homogenate was centrifuged at 500 g, and the resulting supernatant further spun down at 13,000 g for 10 min each. The crude pellet was then suspended in 4 °C storage buffer (SB; 250 mM Sucrose, 5 mM Tris-HCl; pH 7.4). To optimize mitochondrial extraction, the pellet from the first centrifugation step was further hand-homogenized using 12 strokes in ice-cold SB, and centrifuged at 500 g for 10 min. The resulting supernatant was used to re-suspend the crude pellet, mixed gently, and processed for a final centrifugation at 13,000 g for 10 min. This final pellet was suspended in ice-cold SB, with gentle stirring and protected from light until ready to use.

The Bradford mini-assay was used to determine the protein concentration of the final suspension [12]. A protein (BSA) standard curve was generated ranging from 0–12.5 μg.

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Ca^{2+} Uptake and Swelling

Transport of Ca^{2+} into the freshly isolated rat heart mitochondria was determined by a spectrophotometric method using APIII as the Ca^{2+} indicator dye, and monitored at 720 nm [10]. Samples were prepared by adding (as total concentration): energy substrates (5 μM glutamate, 2.5 μM malate), sarcoplasmic reticulum Ca^{2+} inhibitor (0.5 μM thapsigargin), and the endogenous PBR inhibitor (0–10 μM hemin). The desired protein concentration (0.5 mg/ml) was added immediately prior to the assay. A final concentration of 60 μM Ca^{2+} was introduced 30–60 seconds into the assay. A decrease in absorbance values indicated uptake of the Ca^{2+}-APIII complex into the mitochondrial isolates. Ca^{2+} uptake experiments were done with and without cyclosporine A (CsA, 0.2 μM), an inhibitor of MPT.

Protein Purification and Immunoblotting

In order to test the effect of the PBR ligand on VDAC conductance, VDAC-PBR-ANT were isolated and purified from cardiac tissue before fusion in lipid bilayers. Heart mitochondria were isolated from the rat left ventricle according to laboratory protocols, as described above. Protein purification techniques were based from de Pinto et al [13], and modified for our protocols. Briefly, the resulting homogenate was solubilized (10 mM Tris-HCl, 1 mM EDTA, 3 % Triton-X; pH 7) in ice for 30 min. Purification was done by elution of the samples through an equilibrated Q-FF column (GE Healthcare, Piscataway, NJ) using the equilibration buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 % Triton-X; pH 7), washing in increasing series of NaCl (50, 100, 200, 500 mM) + 0.1 % Triton-X, and collecting the eluates. These were then stored in –80 °C until use for lipid bilayer experiments.

The amount of protein in the eluates was detected using the Bradford assay, and determined based on the protein (BSA) standard curve generated. The presence of VDAC and PBR in the purified solutions was in turn determined using the immuno dot blot method [14]. Samples that were immunopositive for VDAC and PBR were separated on a 4–12 % Bis-Tris MES NuPage® gel (Invitrogen, Carlsbad, CA), and transferred to a nitrocellulose membrane. Immunodetection and visualization of VDAC, PBR and ANT bands were done using the WesternBreeze® chemiluminescent protocol (Invitrogen). Antibodies against VDAC (Sigma-Aldrich, St. Louis, MO), PBR (Trevigen, Gaithersburg, MD), and ANT (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1000 dilution, 1–2 hours, at room temperature.

Lipid Bilayer Membrane Experiments

Bilayers were composed of phosphatidylethanolamine: phosphatidylserine:phosphatidylcholine (5:3:2 w/w, Avanti Polar Lipids, Inc, Alabaster, AL) dissolved in decane at a final concentration of 30 mg/ml across a 200 mm aperture on a polysulfone cup (Warner Instrument Corp, CT). The bilayer partitioned two chambers (cis and trans) with buffer solution (in mM) 500 NaCl or CsCl and 20 Hepes-Tris (pH 7.4) on cis, 500 or 50 NaCl or CsCl and 20 Hepes-Tris (pH 7.4) on trans [15]. The addition of protein was made to the cis solution that was held at the virtual ground, whereas the trans solution was connected to the head stage input of an amplifier (Bilayer Clamp BC 525C, Warner Instruments Inc, Hamden, CT). The final concentration of the detergent Triton-X from solubilized protein source was < 0.015 % in the cis chamber. BLM-TC Planar Lipid Bilayer Thermocycler (Warner Instruments) was used to control the recording temperature throughout the experiment at a setting of either 25 °C. After introduction of purified VDAC-PBR-ANT protein complex into cis solution, the induced incorporation of VDAC channels was verified through their voltage signature (ie, exhibiting ionic and anionic currents) and functional characteristics (ie, voltage dependence) [16]. Further incorporation was prevented by extensive perfusion of cis chamber with identical buffer without VDAC-PBR-ANT proteins. The channel gating was monitored and recorded at certain holding potentials from 0, ± 40 to ± 60 mV (applied to the trans side). The amplified current signals, filtered at 1 kHz (Low-Pass Bessel Filter 8 Pole, Warner Instruments) were digitized and acquired at a sampling rate of 10 kHz (Digidata 1320A, Axon-Molecular Devices, Union City, CA). To confirm the effects of hemin on the VDAC-PBR-ANT complex, 2.5, 5 and 10 μM hemin was added to the chamber and conductance observed for at least 3 minutes. Software of pClamp 9.0 was used for analysis and plot without further filtration (Axon-Molecular Devices, Union City, CA). Amplitude histograms were generated using a conventional distribution with a bin width of 0.01 pA and the histogram area was normalized.

Statistical Analysis

Differences between responses at varying concentrations of hemin were analyzed using ANOVA with Holm-Sitek or Dunn's post-test as appropriate on the statistical program SPSS (SPSS, Inc, Chicago, IL). $P < 0.05$ was used as the threshold for statistical significance. Data are presented as mean ± standard error (SE) of the mean.

Results

The PBR Ligand Hemin Reduces Net Calcium Uptake Rate in a Dose-Dependent Manner

Figure 1 shows a representative tracing of extra-mitochondrial calcium kinetics following the addition of 60 μM Ca^{2+} into a solution containing isolated cardiac mitochondria and the calcium-sensitive marker APIII. Since APIII reflects the extra-mitochondrial calcium concentration, a decrease in the absorbance is a result of calcium entry into mitochondria. For analysis of calcium kinetics, the initial uptake rate and amount were measured as indicators of net calcium influx. Calcium release was then quantified as the slope of the APIII signal following loading. Figure 2a shows the net calcium

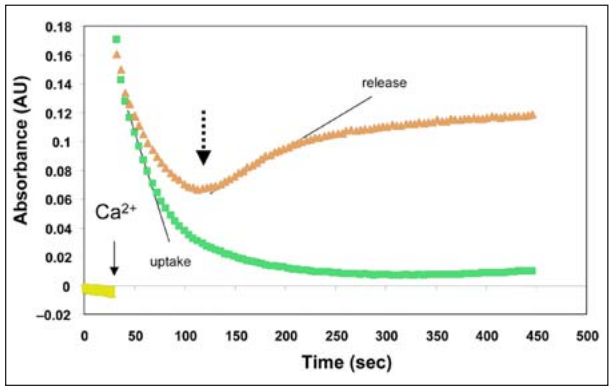

Figure 1. A representative tracing of extra-mitochondrial calcium kinetics following the addition of 60 μM Ca^{2+} into a solution containing isolated cardiac mitochondria and the calcium-sensitive marker APIII. The absorbance (AU) increased rapidly with the addition of Ca^{2+} (solid arrow), followed by a decline representing uptake of calcium by mitochondria. The slope and magnitude of this decline was quantified as calcium uptake rate and amount. Following mitochondrial loading with calcium, an increase in AU reflected calcium release by mitochondria (dashed arrow). Calcium release was quantified as the slope of this line, and was partially inhibited by CsA. The lower trace (no hemin) shows rapid uptake of calcium and an absence of release following loading. The upper trace (hemin = 10 μM) illustrates a reduction in calcium loading as well as early release.

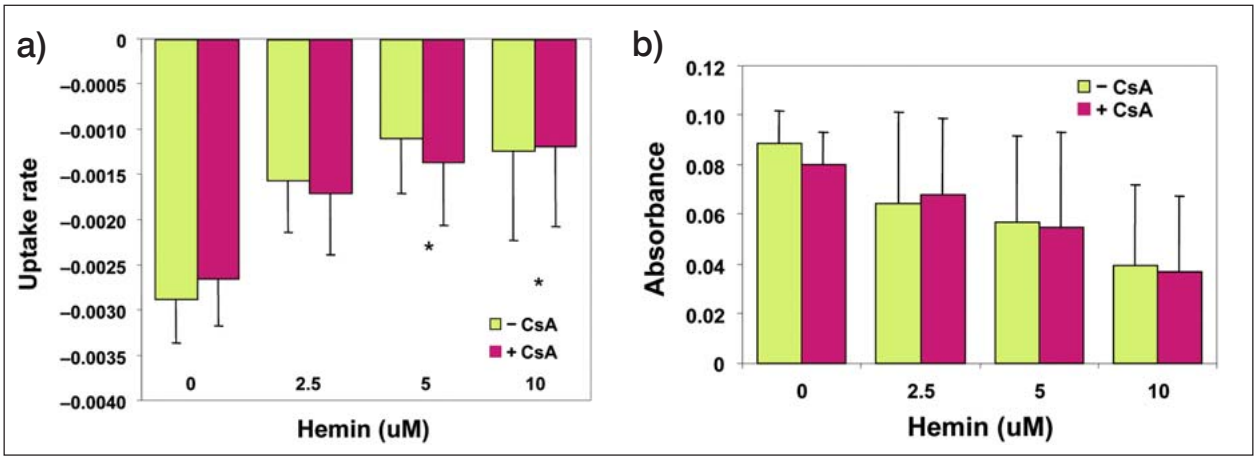

Figure 2. a) Net calcium uptake rate (AU/sec) and **b)** calcium amount (AU) during the initial transport of calcium into mitochondria (uptake in Figure 1) as a function of [hemin] (2.5–10 μM) in the absence and presence of CsA (n = 7). *p < 0.05 vs control.

uptake rate and amount during the initial transport of calcium into mitochondria and demonstrates that there was a dose-dependent reduction in calcium uptake rate over the hemin concentration range of 0–10 μM. The reduction of net calcium amount was similar in magnitude to the reduction in rate; however, these changes did not reach statistical significance due to high variability. A key finding was that CsA did not affect these measurements, indicating that this initial uptake response was independent of the mitochondrial permeability transition.

The PBR Ligand Hemin Results in Dose-Dependent Calcium Release

In control mitochondria in the absence of hemin, mitochondria would rapidly take up calcium and maintain their stores, resulting in a stable external calcium concentration and APIII signal (release slope = 0). These observations indicate that, under the conditions of these experiments, calcium release was not precipitated by exposure to and uptake of a 60 μM Ca^{2+} load. However, exposure to this calcium load in the presence of increasing concentrations of hemin resulted in greater calcium release rates following loading (Fig. 3). This release was significantly inhibited by CsA at hemin 5 and 10 μM that was partially inhibited by CsA, consistent with an important role for permeability transition in this release process.

The PBR Ligand Hemin Reduces VDAC Conductance in a Dose- and Time-Dependent Manner

Using the techniques described above, VDAC-PBR-ANT co-precipitates and PBR alone were purified from heart mitochondria samples. VDAC alone could not be isolated, as each preparation with VDAC also contained PBR. Eluates enriched in VDAC-PBR-ANT were then diluted and applied to cis chamber (having a 10:1 chemical gradient against the trans side of bilayer membrane) to induce channel incorporation into lipid bilayers [15]. The currents through the reconstituted channel(s) were recorded under certain transmembrane holding potentials and hemin concentrations. The results demonstrate that hemin at 5 or 10 μM reduced VDAC voltage-dependent conductance, particularly after a 3-minute exposure (n = 7). Figure 4 shows a representative experiment, demonstrating that, in the absence of hemin, the channels exhibited typical behavior characteristic of VDAC,

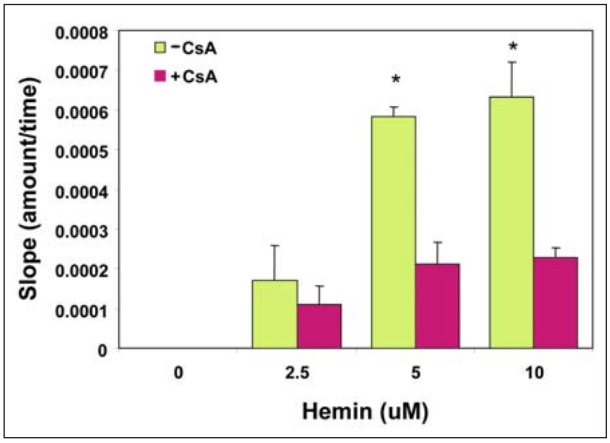

Figure 3. Rate of calcium release (AU/sec) from isolated mitochondria as indicated by an increase in APIII absorbance signal following calcium loading in the absence and presence of CsA under different hemin concentrations (n = 7). There was no calcium release under control conditions in the absence of hemin. Calcium release was significantly greater than control in all experiments using hemin 2.5–10 μM with or without CsA (p < 0.01). In addition, calcium release was greater without CsA than with CsA at hemin 5 and 10 μM (*p < 0.05).

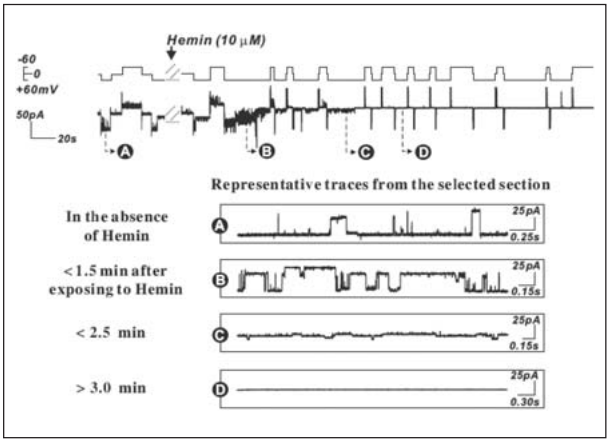

Figure 4. Representative current traces of VDAC-PBR-ANT channel complex in lipid bilayers. Under control conditions, VDAC-PBR-ANT isolates exhibited characteristic changes in conductance with applied voltages (Section A). Following exposure to hemin 5–10 μM, there was gradual disappearance of conductance changes, resulting in complete channel closure after ~ 3 min (n = 7). The top trace depicts the voltage protocol used for the corresponding current.

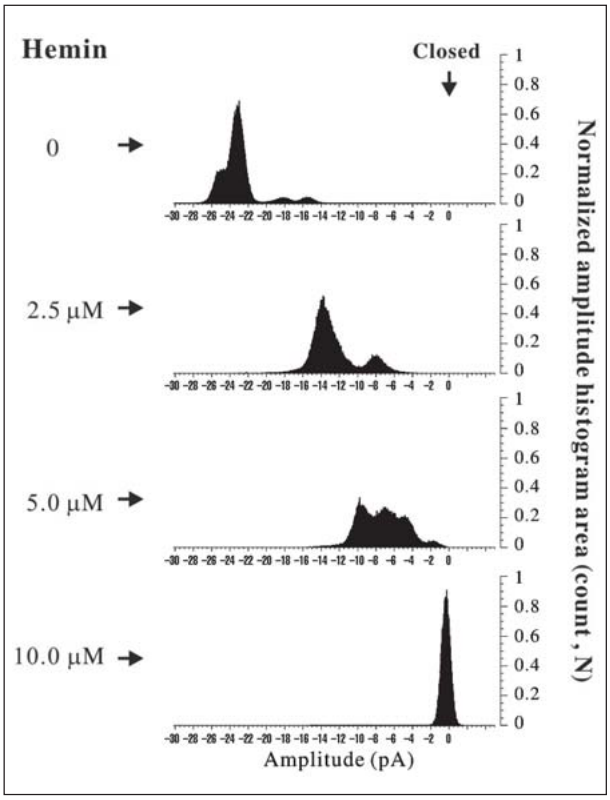

Figure 5. Current amplitude histograms of a VDAC-PBR-ANT channel complex reconstituted in lipid bilayer as exposure to defined concentrations of hemin. A the holding potential of –40 mV (on trans), VDAC-PBR-ANT stayed more frequently at maximal open state. Addition of 2.5, 5 and 10 μM hemin in the cis chamber promoted reduced current amplitudes to a lower and eventually zero value.

namely a rapid response to a change of the transmembrane potentials – fluctuating between multiple sub-conductance states (Fig. 4, Section A). Sections B, C, and D of Figure 4 display the current traces of the channel at ~1.5 min, ~2.5 min and > 3 min after addition of hemin (10 μM final in cis solution). As expected, no obvious current was detected through intact bilayer in the presence of PBR alone (n > 10).

Figure 5 shows the current distribution of a VDAC-PBR-ANT channel in the absence and the presence of 2.5 and 10 μM hemin, respectively. As expected, at a holding potential of –40 mV (on trans) without hemin in the solution, the distribution of current amplitude was more toward maximal level (~18–22 pA, left histogram panel). However, upon the addition of 2.5 μM hemin, the current distribution shows more frequent occupancy in the lower and zero (closed state) areas (middle histogram panel). The final panel (right histogram) shows that 10 μM hemin drastically stabilized the channel at a completely closed state with a current level that was mostly zero.

Discussion

This study presents several key findings supporting the hypothesis that PBR has a regulatory role on VDAC-modulated entry of Ca^{2+} into heart mitochondria. First, the endogenous PBR ligand hemin was able to reduce initial uptake of calcium into mitochondria in a dose-dependent manner that was independent of the permeability transition. Second, mitochondria exposed to a calcium load in the presence of hemin subsequently released calcium following loading, a response partially inhibited by CsA. Third, the effects of hemin on the VDAC-PBR-ANT complex were inhibitory and dose-dependent when tested in lipid bilayers. Together, these data indicate a potentially important regulatory role for the 18 kDa peripheral benzodiazepine receptor on VDAC function and, as a consequence, on metabolite and ion transport into and out of mitochondria.

VDAC

The voltage-dependent anion channel (VDAC) is found in mitochondria of all eukaryotic organisms. This channel can exist in a variety of functional states which mediate its permeability to anions, cations and metabolites. Important compounds transported through VDAC include ATP, NADH, and Ca^{2+}, indicating that VDAC function has an important regulatory role in transport across the outer mitochondrial membrane (OMM) [1]. The permeability of VDAC is determined not only by membrane voltage, but also by its conductance state determined, in part, by associated regulatory proteins [17, 18]. These proteins include the adenine nucleotide translocator (ANT) and the mitochondrial peripheral benzodiazepine receptor (PBR), which form a complex with VDAC to regulate transport across the OMM. Another important function of VDAC is its central role in the formation of the mitochondrial permeability transition (MPT) [19]. The MPT, in a manner similar to VDAC in its inward transport function, is generally considered to be an assembly of proteins including VDAC, ANT, PBR, hexokinase, creatine kinase, Bax/Bcl-2, and cyclophilin-D which functions to release mitochondrial compounds and promote apoptosis [20]. The essential role that VDAC plays in the transport of crucial compounds across the mitochondrial membranes makes knowledge of its regulation important.

There is substantial evidence that calcium transport across the mitochondrial membrane is modulated by VDAC. In liver mitochondria, Gincel et al [5] showed that VDAC was permeable to Ca^{2+}, has Ca^{2+} binding sites, and is inhibited by ruthenium red (an inhibitor of the calcium uniporter). In fact, the data support the concept that the VDAC-PBR-ANT complex may be the calcium uniporter, the primary entry pathway for calcium in mitochondria. In support of this concept, Rapizzi et al [21] demonstrated that VDAC over-expression increased mitochondrial Ca^{2+} loading and apoptosis.

Mitochondrial Peripheral Benzodiazepine Receptor (PBR)

As noted above, the PBR is an 18 kDa protein associated with VDAC that can modulate VDAC conductance and, hence, mitochondrial function under a variety of physiologic and pathophysiologic conditions [18]. Despite clear evidence of the association of the PBR with VDAC, the mechanism of PBR modulation of VDAC is unknown. However, ligands of the PBR have demonstrated an ability to precipitate MPT and apoptosis [22, 23], presumably by their interaction with the PBR component of the MPT. Conversely, some ligands (eg, SSR180575 or Ro5-4864) are protective under conditions of ischemia/reperfusion, although the mechanism of this effect is unknown [24].

The characteristics of PBR ligands and their binding sites vary, thereby resulting in potentially different effects on membrane transport and MPT. The ligand used in this study, hemin or protoporphyrin IX (PPIX), is an endogenous PBR ligand involved in the intracellular transport of porphyrins and heme which has been shown to have high affinity for the PBR (Kd ~12–15 nM) [25, 26]. Hemin was selected over pharmacologic ligands because of its key role in physiologic processes [25], as well as data showing that hemin may be protective in a model of cardiomyopathy [27].

Hemin Limits Calcium Overload in Isolated Mitochondria

Upon exposure to a calcium load, mitochondria take up calcium, a process completely inhibited by ruthenium red, as shown in prior studies [5] and the current study. We postulated that, given the critical role of VDAC in Ca^{2+} transport, modulation of the PBR by an endogenous ligand would limit calcium uptake. The current data support this concept, since hemin limited initial Ca^{2+} uptake in a dose-dependent manner. This effect was seen both in the rate of calcium uptake, as well as in its amount (Fig. 2). Since calcium overload is likely a key element in mitochondrial and cellular death, either through necrosis or apoptosis [28], the effect of hemin limiting calcium entry at low concentrations is a potential therapeutic intervention similar to that of inhibiting calcium entry using Ru360 [9]. This effect may help explain the beneficial results seen when hemin was administered to rats with adriamycin-induced cardiomyopathy [27]. It should be noted, however, that hemin has dual actions on calcium transport, particularly at higher concentrations (≥ 5 μM). As seen in the current experiments, and as previously seen in isolated rat brain mitochondria [29], hemin both inhibits Ca^{2+} uptake and promotes permeability transition.

Hemin Effects on VDAC/PBR Conductance in Lipid Bilayers

Consistent with the known association of PBR and VDAC, hemin decreased conductance through VDAC as reflected in significantly reduced current through the reconstituted channels in bilayers. The effect of hemin on calcium uptake in isolated mitochondria is consistent with this interaction, as is the effect of hemin on permeability transition [30]. Unknown at this time is the mechanism of PBR modulation of VDAC. Potential interactions between PBR and VDAC include phosphorylation of a 43 kDa protein involved in electron transport [31] leading to changes in membrane voltage sensitive VDAC sensor and/or binding of PBR to a mobile N-terminal amphipathic α-helix arm [32] resulting in conformational change in the VDAC barrel.

Limitations

These experimental results should be placed in the context of the model used. First, they were performed using mitochondria and proteins isolated from rat cardiac tissue. As such, the applicability of these findings to in-vivo models or other species may be limited. Second, the concentrations of hemin used were significantly higher than the reported K_i of 15–40 μM [33], opening the possibility that non-specific effects could account for some of the findings. However, the dose-response from 2.5–10 μM demonstrated minimal effects at the lowest concentration and the bilayer experiments also showed dose-dependent inhibition at these concentrations. Together, these observations suggest that any non-specific effects of hemin, in this model, were unlikely to have a significant role. Third, the calcium concentration used to assess calcium uptake was higher than reported $[Ca^{2+}]_i$ during reperfusion [7]. However, intact mitochondria without hemin were able to take up calcium at this exposure without precipitating permeability transition (as measured by calcium loss), indicating that this exposure resulted in a physiologic response. Fourth, the experiments using isolated mitochondria could not distinguish the relative roles of a direct effect of hemin on PBR (and hence the VDAC-PBR-ANT complex) versus an indirect effect of hemin on membrane potential and therefore on VDAC conductance. Finally, the classic description of the MPT as an assembly of proteins including VDAC, ANT, PBR, hexokinase, creatine kinase, Bax/Bcl-2, and cyclophilin-D which functions to release mitochondrial compounds and promote apoptosis [20] had recently been challenged by experiments using VDAC knockout mice and showing preservation of some MPT-related functions [34]. Thus, the effect of hemin on CsA-inhibited calcium release may involve other effects beyond those on VDAC.

Conclusion

In conclusion, these experiments have demonstrated an important role for the PBR ligand hemin in modulating VDAC conductance in lipid bilayers and calcium transport in isolated mitochondria. The effects of hemin are complex, with initial inhibition of calcium transport into mitochondria and subsequent release of calcium, in part through permeability transition. Since bilayer experiments indicate a direct effect of hemin on VDAC conductance, the inhibition of calcium entry is likely due to direct PBR modulation of calcium entry through VDAC, although an indirect effect of hemin on membrane potential and VDAC conductance cannot be excluded. The dual effects of hemin on calcium entry and permeability transition may be due to the presence of both low and high affinity binding sites of the ligand [6], or the presence of different associated proteins involved in either calcium entry (eg, mitochondrial uniporter) or release (permeability transition).

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