Differential Effects of Mitochondrial Heat Shock Protein 60 and Related Molecular Chaperones to Prevent Intracellular \(\beta\)-Amyloid-induced Inhibition of Complex IV and Limit Apoptosis*

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Defects in mitochondrial oxidative metabolism, in particular decreased activity of cytochrome \(c\) oxidase, have been reported in Alzheimer disease tissue and in cultured cells that overexpress amyloid precursor protein. Mitochondrial dysfunction contributes to neurodegeneration in Alzheimer disease partly through formation of reactive oxygen species and the release of sequestered molecules that initiate programmed cell death pathways. The heat shock proteins (HSP) are cytoprotective against a number of stressors, including accumulations of misfolded proteins and reactive oxygen species. We reported on the property of Hsp70 to protect cultured neurons from cell death caused by intraneuronal \(\beta\)-amyloid. Here we demonstrate that Hsp60, Hsp70, and Hsp90 both alone and in combination provide differential protection against intracellular \(\beta\)-amyloid stress through the maintenance of mitochondrial oxidative phosphorylation and functionality of tricarboxylic acid cycle enzymes. Notably, \(\beta\)-amyloid was found to selectively inhibit complex IV activity, an effect selectively neutralized by Hsp60. The combined effect of HSPs was to reduce the free radical burden, preserve ATP generation, decrease cytochrome \(c\) release, and prevent caspase-9 activation, all important mediators of \(\beta\)-amyloid-induced neuronal dysfunction and death.

Current evidence indicates that intracellular accumulation of \(\beta\)-amyloid in neurons leads to the formation of an insoluble pool in which the peptide is highly aggregated as well as a soluble pool in which toxic oligomers are found (1–3). \(\alpha\beta\)-(1–42) is generated in both the endoplasmic reticulum and the intermediate compartment (4). Because neuronal mitochondria are frequently found in close association with the endoplasmic reticulum (5), it is possible that the local concentration of \(\alpha\beta\) is relatively higher in their vicinity compared with other cellular locations. Conceivably, exposure of mitochondria to \(\alpha\beta\) alters membrane stability and/or their normal oxidative phosphorylation functions. These considerations make it feasible that a direct impact of intracellular \(\beta\)-amyloid on mitochondrial function may contribute to neurodegeneration in AD through energy failure and activation of apoptosis.

A recent study of mitochondrial function in a transgenic model and in AD patients has shown that oxidative phosphorylation is inhibited by the presence of intracellular \(\beta\)-amyloid (6), leading to a reduction in ATP production. ATP depletion leads to partial membrane depolarization, release of the voltage-dependent Mg\(^{2+}\) block of NMDA receptors, increase in calcium influx, and a decrease in calcium buffering capacity (7). The increase in intracellular calcium enhances free radical generation and triggers several pathways that lead to cellular dysfunction and death (8). Examination of brains from patients suffering from AD identifies a high fraction of cells undergoing apoptosis, which is suggested as an important mechanism for neuronal cell loss (9). One of the major events related to activation of the intrinsic programmed cell death pathway is the release of mitochondrial factors, e.g. cytochrome \(c\) into the cytoplasm (10).

Increasing evidence suggests a potential involvement of specific molecular chaperones in several neurodegenerative diseases (11–13). Recent studies show that heat shock proteins are able to suppress neurotoxicity in animal models of Parkinson and polyglutamine disorders (14–18). Mammalian HSPs have been classified into two groups according to their size, namely high molecular weight HSPs and small HSPs (19, 20). As they relate to neurodegenerative conditions, Hsp70 and Hsp90 have been implicated in the maintenance of tau solubility and the suppression of tau aggregates (21). We have previously shown that Hsp70 is induced by neurons in an attempt to suppress intracellular \(\alpha\beta\)-mediated toxicity and that engineered overexpression of Hsp70 can limit this toxicity (22). The mitochondrial chaperons, composed of heat shock protein 60 (Hsp60) and 10 (Hsp10), are primarily found in the mitochondrial matrix, where they participate in the refolding of misfolded mitochondrial proteins. The mitochondria are a specific target of the protective effect of heat shock specifically against oxidative injury (23).

The abbreviations used are: A\(\beta\), \(\beta\)-amyloid; AD, Alzheimer disease; HSP, heat shock protein; KGDH, \(\alpha\)-ketoglutarate dehydrogenase; MOPS, 4-morpholinepropanesulfonic acid; PDH, pyruvate dehydrogenase; LDH, lactate dehydrogenase; Bis-Tris, 2-[bis(2-hydroxyethyl)aminom]-2-(hydroxyethyl)-propane-1,3-diol; MES, 4-morpholineethanesulfonic acid; ETC, electron transport chain; Dax, doxycycline; Ad, adenosvirus; DCF, 2,7'-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species.

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The activation of heat shock gene transcription is mediated by heat shock transcription factor, which binds to heat shock elements in their respective promoter regions (24–26). Heat shock transcription factor 1 is found to be a primary component of the heat shock element binding activity in cells exposed to heat shock and many other physiological stresses (27–29). Hosokawa et al. (30) reported that specific flavonoids, including quercetin, inhibited the transcription of various HSPs after heat shock and that this effect was due to the inhibition of heat shock transcription factor activation.

The possible connection between intracellular Aβ cytotoxicity and mitochondrial dysfunction suggests a strategy to protect susceptible mitochondrial functions by using molecular chaperones. In the present study we have examined the functional role of several HSPs to protect vital mitochondrial enzymes and respiratory chain components against poisoning caused by intracellular Aβ. An inducible adenovirus system was used to deliver Aβ-(1–42) to neuroblastoma cells and primary cortical neurons, which had previously been infected with adenovirus encoding the expression of Hsp60, -70, and -90. We were able to demonstrate that the expression of these proteins was from Sigma.

Reagents and Antibodies—The following antibodies were used: 6E10 (1:300; Signet Laboratories, Dedham, MA), anti-HSP70 (1:100–5000; Stressgen Biotechnologies), anti-Hsp60 (1:5000; Stressgen Biotechnologies), and anti-Hsp60 (1:100; Stressgen Biotechnologies). All other chemicals and reagents used: 6E10 (1:300; Signet Laboratories, Dedham, MA), anti-Hsp60 (1:100; Stressgen Biotechnologies), and anti-Hsp60 (1:100; Stressgen Biotechnologies).—Anti-Hsp60 (1:5000; Stressgen Biotechnologies). All other chemicals and reagents were from Sigma.

Cell Culture—Human neuroblastoma SH-SY5Y cells were grown to 70% confluence in Dulbecco’s modified Eagle’s medium (Invitrogen) plus 10% (v/v) heat-inactivated fetal bovine serum (Sigma), antibiotics, and 2 mM l-glutamine at 37 °C and 5% CO₂. For primary cortical neurons, cortices from 2-day-old mouse pups were isolated and trypsinized, and the cells were dissociated by repetitive trituration through a Pasteur glass pipette, counted (1500 cells seeded/mm²), and grown at 37 °C and 5% CO₂ in neurobasal medium (Invitrogen) supplemented with 2% (v/v) B-27 supplement (Invitrogen).

Virus-based Expression—Adenoviruses encoding HSP, LacZ, and Aβ-(1–42) as previously described (22) were grown to high titer in human embryonic kidney 293 cells and purified by cesium chloride density gradient ultracentrifugation. Viral titer was determined by plaque assay. Cell cultures were infected with Adenovirus Hsp60, Hsp70, and Hsp90 for 24 h. The medium was replaced, and cultures were co-infected with AdTet-On and AdTRE-Aβ-(1–42) for 24 h at a 1:5 ratio (100 multiplicity of infection). Doxycycline-inducible adenoviral construct encoding (AdTRELaCZ) bacterial β-galactosidase and the positive regulator virus (AdTet-On) in a 5:1 ratio infection served as controls. Unless otherwise indicated, transgene expression was induced after adding 1 μg/ml doxycycline for 24 h. Functional activity and/or-expression of HSPs was inhibited after adding 100 μM Quercetin (Sigma-Aldrich) 4 h before and again 6 h after induction with doxycycline.

Mitochondrial Isolation—Cells were washed and resuspended in ice-cold sucrose-mannitol buffer followed by homogenization in a Dounce homogenizer. Homogenates were centrifuged at 600 × g for 5 min at 4 °C. The supernatant containing the mitochondria was saved; the pellet was resuspended in isolation buffer and rehomogenized. Centrifugation of the resuspended homogenate was repeated, and the supernatants were pooled. The pooled supernatant was centrifuged at 16,000 × g for 10 min at 4 °C. The final pellet containing the mitochondria was resuspended in 500 μl of isolation buffer for determining the activities of oxidative phosphorylation and tricarboxylic acid cycle enzymes. Mitochondrial protein determinations were performed on diluted samples using the microscale version of the Bio-Rad protein assay kit. 10 μg of mitochondrial protein from each sample was used in subsequent experiments (Figs. 2–4 and 6). The high speed supernatant, consisting of the cytosolic compartment, was probed for cytochrome c release and caspase-9.

Complex I—Activity was determined in homogenates of isolated mitochondria as rotenone-sensitive NADH dehydrogenase-mediated reduction of 2,6-dichlorophenolindophenol (DCPIP). Isolated mitochondrial extract was freeze-thawed in hypotonic medium. The addition of 200 μM NADH, 200 μM decylubiquinol, 2 mM KCN, and 0.002% DCPIP to the extract in the presence and absence of 2 μM rotenone started the reaction, which was monitored at 600 nm at 30 °C.

Complex II-III—Activity was determined in isolated mitochondrial preparations. Mitochondrial samples were added to buffer containing 50 mM Tris-SO₄ (pH 7.4), 100 mM EDTA, 2 mM succinate, and 1% dodecylmaltoside. The assay was initiated with the addition of 100 mM cytochrome c and followed by the absorbance, measured at 550 nm at 30 °C in the presence and absence of 4 mM antimycin.

Complex IV—Activity was determined in isolated mitochondrial preparations. Mitochondrial samples were added to buffer containing 10 mM KPO₄ (pH 7.4), 100 mM KCl, 0.025% dodecyl maltoside, and 80 mM ferrocytochrome c. The addition of cytochrome c was used to initiate the reaction. Incubation with KCN inhibited complex IV activity. Absorbance was measured at 550 nm at 30 °C. One unit of complex IV activity will oxidize 1.0 μmol of ferrocytochrome c/min at pH 7.0 and 25 °C.

α-Ketoglutarate Dehydrogenase (KGDH)—Activity was assayed as the rate of NAD⁺ reduction at 340 nm upon the addition of 5.0 mM MgCl₂, 40 μM rotenone, 2.5 mM α-ketoglutarate, 1.0 mM CoA, 0.2 mM thymine pyrophosphate, and 1.0 mM NAD⁺. The addition of mitochondrial sample was used to initiate the reaction. One unit of KGDH activity will convert 1.0 μmol of β-NAD to β-NADH/min.

Pyruvate Dehydrogenase Assay—PDH activity was determined as the rate of NAD⁺ reduction at 340 nm upon the addition of (final concentrations) 51 mM MOPS, 0.20 mM magnesium chloride, 0.01 mM calcium chloride, 0.30 mM carnitine, 0.12 mM coenzyme A, 2.0 mM β-NAD, 2.64 mM 1-cysteine hydrochloride, and 5.1 mM pyruvate in a 1-ml reaction mix. The addition of mitochondrial sample was used to initiate the reaction. One unit of PDH activity will convert 1.0 μmol of β-NAD to β-NADH/min in the presence of saturating levels of coenzyme A.
Hsp60 Protects against Aβ-induced ETC Inhibition

Polarography Measurement of Respiratory Complex Activity in Mice Cortical Neurons—The mitochondrial fraction from primary cortical neurons was resuspended in respiration buffer (100 mM KCl, 75 mM mannitol, 25 mM sucrose, 10 mM Tris phosphate (pH 7.4)) and stored on ice before measurement of respiration rate. Oxygen consumption rates are measured polarographically using an oxygraph (HanseTech Instruments Ltd, Norfolk, England) in a water-jacketed chamber. The reaction volume of 500 μl was maintained at 30 °C. The electrode was connected to a PC running Oxygraph Plus™ software. 10 μl of mitochondrial suspension was added to the chamber to a final concentration of 0.4 mg of protein/ml. Final concentrations of 10 mM glutamate and 5 mM malate were added as substrates for complex I. Complex III respiration was supported by the addition of 10 mM succinate. Complex IV respiration was supported by the addition of 10 mM ascorbate, 200 μM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD). The activities were calculated as the fraction sensitive to the respective inhibitors; rotenone for complex I, antimycin for complex II, and potassium cyanide for complex IV.

Measurement of ATP Production—ATP levels were determined as described by Manfredi et al. (31). To test the mitochondrial component of ATP synthesis, 5 mM oligomycin (ATP synthase inhibitor) was added to freshly isolated mitochondria and incubated for 10 min at 37 °C. The inhibition of ATP production was determined by subtracting the oligomycin-treated preparation from identical untreated sample.

H2O2/Reactive Oxygen Species (ROS) Production—To measure reactive oxygen species, cells were suspended in 1 ml of Dulbecco’s modified Eagle’s medium containing 50 μM 2',7'-dichlorodihydrofluorescein diacetate (DCF; Molecular Probes, Eugene, Oregon) for 45 min at room temperature. Cells were washed twice with phosphate-buffered saline and lysed by sequential freezing/thawing in water. After homogenization the lysates were cleared by centrifugation, and DCF oxidation was measured at 525 nm (excitation at 475 nm) in a microplate reader (CytoFluor 2350, Perseptive).

Lactate Dehydrogenase Release Assay—Conditioned cultured medium was clarified by centrifugation, and LDH enzyme activity measured using the BioVision LDH cytotoxicity assay kit (BioVision, Inc., Mountain View, CA) according to the manufacturer’s protocol. Test media were assayed in triplicate for LDH activity measured using the BioVision LDH cytotoxicity assay kit and incubated for 10 min at 37 °C. The inhibition of ATP production was determined by subtracting the oligomycin-treated preparation from identical untreated sample.

Heat Shock Experiments—SH-SY5Y cells normally maintained at 37 °C were first preconditioned at 42 °C for 15 min in 5% CO2. Thereafter, heat shock was induced at 45 °C for 30 min. After heat shock cultures were allowed to recover for 2 h at 37 °C. Cells were lysed with Triton X-lysis buffer, and cell extracts were used for Western blot analysis of caspase-9 products.

Protein Extractions, Electrophoresis, and Western Blotting—For routine whole cell lysates, cultures were extracted with radioimmune precipitation assay buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS plus protease inhibitors (Complete Protease inhibitor; Roche Applied Science). Protein determinations were performed on diluted samples using the microscale version of the Bio-Rad protein assay kit. For Western blotting proteins were electrophoretically separated and subsequently transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). After the incubation with specific, primary antibodies, blots were exposed to secondary antibody conjugated to horseradish peroxidase (DAKO/Cytomation, Carpinteria, CA). The blots were developed using enhanced chemiluminescence reagents and film from Amersham Biosciences. For analysis of amyloid levels, samples were fractionated on 4–12% Bis-Tris gels using MES running buffer (Invitrogen). For cytochrome c and caspase-9 level determinations, 4–20% Tris-glycine gels (Bio-Rad) were utilized.

Statistical Analysis—The data were expressed as mean ± S.D. based on data derived from five independent experiments. Statistical analysis was performed using Graphpad Prism® Software, and statistical significance was tested by one-way analysis of variance and when appropriate followed by Bartlett’s test for equal variances.

RESULTS

We first tested the hypothesis that accumulation of intracellular β-amyloid leads to increased production of ROS and energy depletion. Then we examined whether directed expression of Hsp60, Hsp70, and/or Hsp90 would provide protection against these insults. SH-SY5Y human neuroblastoma cells were infected individually (or in combination) with adenoviral constructs encoding Hsp60, Hsp70, and Hsp90. 36 h after these infections cells were co-infected with a previously validated (22) doxycycline-inducible adenoviral construct encoding human Aβ-(1–42) (AdTREA-(1–42)). Alternatively, cells were induced to express the inert protein, β-galactosidase (AdTRELaZ). In either case the positive regulator virus AdTRETet-on was coinfected in a 5:1 ratio. 24 h after this infection, Aβ or Lac Z expression was induced by the addition of 1 mg/ml of doxycycline for 24 h. Uninduced cultures and LacZ-expressing cultures served as controls. The data in Fig. 1 demonstrate that the adenoviral systems we employ are competent to direct the expression of the respective heat shock proteins as well as β-amyloid monomer and oligomer in SH-SY5Y cells. Notably, overexpression of Hsp60 did not appear whatsoever to alter the steady-state levels of Aβ (Fig. 1, a, lane 4 versus 6 and e, bars 1 and 2). Hsp70 overexpression slightly attenuated steady-state Aβ levels (~25%) in steady-state Aβ levels in SH-SY5Y cells as compared with controls (Fig. 1, b, lane 4 versus 6, and e, bar 3 versus 4). Hsp90 expression led to a more significant decrease (~45%) (Fig. 1, c and e, bar 5 versus 6). Aβ expression had no effects on any of the endogenous HSP levels (Fig. 1, a, b, and c, lanes 6 versus 1 and 2). Viral-encoded control viral expression (LacZ) had no effect on endogenous (or expressed) HSP levels, and HSP overexpression in turn had no effect on control LacZ levels (Fig. 1d, lanes 6 versus 1 and 2, lane 4 versus 3 and 5, and lane 4 versus 6). ROS were measured using the conversion of 2',7'-dichlorodihydrofluorescein to the fluorescent compound DCF. Aβ accumulation in SH-SY5Y cells led to a significant increase in the production of ROS and a concomitant reduction in ATP synthesis (Fig. 2, a and b; bar 2). Levels of ROS in the presence of β-amyloid accumulation were significantly reduced by the individual expressions of either Hsp60 or Hsp70 (Fig. 2a, bars 3 and 4). Hsp90 expression was not as effective in achieving such reduction (Fig. 2a; bar 5). Co-expression of all three HSPs did...
Hsp60 Protects against Aβ-induced ETC Inhibition

not lead to any additional protective effects on ROS reduction. Interestingly, this same experimental paradigm yielded a different outcome in terms of cellular ATP biosynthesis (Fig. 2b). Although intracellular Aβ expression led to an ~50% reduction in ATP biosynthetic capacity, the effect was ameliorated only by the expression of Hsp60 (bar 3). No response was obtained with expression of either Hsp70 or Hsp90. When all three Hsps were co-expressed, the slightly greater preservation of ATP synthesis appeared mainly attributable to Hsp60 (Fig. 2b, bars 6 versus 3, 4, and 5). Control virus expression AdTRELacZ with or without HSPs had no effect on ATP production (Fig. 2b, bar 7 versus 8).
Hsp60 Protects against Aβ-induced ETC Inhibition

ATP production in the mitochondria is obligately coupled to oxidative phosphorylation through the electron transport chain. Intracellular Aβ-(1–42) expression had no effect on either complex I activity (NADH-ubiquinone oxidoreductase) or complex II + III (succinate dehydrogenase + succinate-cytochrome c reductase) activity in SH-SY5Y cells (Fig. 3a). Complex IV activity on the other hand was significantly impaired by the accumulation of Aβ as compared with control cells (Fig. 3b, bar 2 versus 1). Overexpression of Hsp60 was significantly protective toward complex IV activity against the Aβ-toxicity (Fig. 3b, bar 3 versus 2), whereas Hsp70 and Hsp90 provided little or no benefit in this regard. The mechanism behind the specific toxic effect of intracellular Aβ on complex IV activity involves a change in the apparent $K_m$ (Table 1) of cytochrome oxidase for reduced cytochrome $c$ as compared with controls. Hsp60 overexpression significantly prevented this inhibition, whereas Hsp70 and Hsp90 did not. No significant additive protection against the intracellular Aβ-mediated effect was observed with the addition of Hsp70 and Hsp90 to Hsp60 (Fig. 3b, bar 6).

It has been proposed that Aβ exerts its deleterious effects on mitochondrial enzyme complexes by first increasing the production of free radicals, which in turn cause damage to mitochondrial membranes and protein subunits. After induction of intracellular Aβ, we find a significant inhibition of two key mitochondrial enzyme complexes, PDH and α-KGDH (Fig. 4), which are responsible to provide the major reducing substrate to the electron transport chain (ETC). Using the same interventions as described above, we overexpressed Hsp60, -70, and -90 alone and in combination and measured the effects on restoration of enzyme activity for both of these complexes. The overexpression of Hsp60 alone conferred the prevention of Aβ-induced loss of PDH enzyme complex activity, the entry point for the tricarboxylic acid cycle (Fig. 4a). This effect was not seen with either Hsp70 or -90. Interestingly, in the attempt to prevent Aβ-induced loss of α-KGDH activity, Hsp60 and -70 were essentially ineffective, whereas Hsp90 proved robust (Fig. 4b, bar 5 versus 2).

Quercetin has been shown to interfere with the activity and/or expression of heat shock proteins in numerous settings (29, 30). We investigated the effects of this bioflavonoid on endogenous HSP expression in primary cortical neurons in the presence or absence of added stress from the accumulation of intracellular β-amylloid. We did not find any steady-state inhibition of HSP expression in the absence of Aβ-oxidative stress with the addition of 100 mM quercetin (Fig. 5a, lane 4 or 5 versus 3).

![Figure 2](image1.png)

**FIGURE 2.** a, ROS is estimated utilizing a converting reaction of the probe 2',7'-dichlorodihydrofluorescein to fluorescent DCF. After Aβ-(1–42) expression, the levels of oxygen radicals rose significantly (bar 2) compared with control cells (bar 1) without Aβ expression. Increased expression of Hsp60 or Hsp70 (**, $p < 0.01$), but not Hsp90, lowered free radical generation in cells that express intracellular Aβ. The combination of all the three, Hsp60, Hsp70, and Hsp90, did not provide an additive effect (bar 6). b, ATP generation in SH-SY5Y cells producing HSPs alone and in combination. ATP generation significantly drops in Aβ-expressing cells (bar 2). Hsp60 alone appears to be most efficient in counteracting β-amyloid (bar 3). An additive effect to raise ATP production is shown in combination with Hsp70 and Hsp90 (bar 6), $n = 5$ in each case. Control LacZ expression did not alter ATP levels (**, $p < 0.01$) compared with control; *, $p < 0.05$ compared with β-amyloid stressed group. Error bars ± S.D. U, units.

![Figure 3](image2.png)

**FIGURE 3.** a, amyloid expression did not alter activity levels of complex I or complex II + III in SH-SY5Y cells. b, induction of β-amyloid significantly inhibited the complex IV activity compared with control cells (bar 2). Overexpression of Hsp60 alone nearly reversed this inhibition. $n = 5$ in each group. ***, $p < 0.01$ compared with control; **, $p < 0.05$ compared with β-amyloid stressed group. Error bars ± S.D. U, units.
Hsp60 Protects against Aβ-induced ETC Inhibition

TABLE 1

| Condition                   | $K_m$  | $V_{max}$ | $K_m$  | $V_{max}$ |
|-----------------------------|--------|-----------|--------|-----------|
| AdTREAB + AdTet-On (Control)| 11.17  | 0.1786    | 3.22   | 0.01124   |
| AdTREAB + AdTet-On + Dox   | 36.68  | 0.1799    | 9.48   | 0.01659   |
| AdHsp60 + AdTREAB + AdTet-On + Dox | 15.7   | 0.1743    | 1.58   | 0.00470   |
| AdHsp70 + AdTREAB + AdTet-On + Dox | 29.4   | 0.1753    | 9.08   | 0.0164    |
| AdHsp90 + AdTREAB + AdTet-On + Dox | 13.72  | 0.1728    | 1.64   | 0.00508   |
| AdHsp60/70/90 + AdTREAB + AdTet-On + Dox | 11.17  | 0.1786    | 3.22   | 0.01124   |

*p < 0.01 compared to control; n = 5.

*p < 0.01 and compared to β-amyloid stressed group. $K_m$ = ½ the maximum substrate concentration; n = 5.

FIGURE 4. a, overexpression of Hsp60 alone significantly protected the PDH complex, the entry point of the tricarboxylic acid cycle, by increasing the activity of the enzyme compared with the virus-alone control group (bar 3 versus 2). b, Hsp90 alone significantly improved α-KGDH activity (bar 5) compared with virus alone control group (bar 2). Simultaneous overexpression of all three HSPs offered a slight boost to both PDH and α-KGDH activities (bar 6). n = 5 in each group. #, p < 0.05 compared with control; *, p < 0.05 compared with β-amyloid stressed group. Error bars ± S.D. U, units.

I) and accompanying bar sets. Western blot and densitometry analyses of neuronal extracts show that expression of Aβ leads to a surprisingly substantial reduction in Hsp60 levels (Fig. 5a, lane and bar set 3). No change or even a slight induction is observed in levels of Hsp70 or Hsp90 (Fig. 5a, lane and bar set 3 versus 2). The addition of quercetin contemporaneously with viral-mediated Aβ expression led to a significant enhancement in the accumulation of steady-state Aβ in infected cortical neurons (Fig. 5a, lane and bar set 6 versus 3). Moreover, this increase in amyloid load, especially in the presence of quercetin, was consistently associated with the complete loss of Hsp60 signals as well as significant reduction in endogenous Hsp70.

We next tested if overexpression of all HSPs would reverse the quercetin effect to increase Aβ levels. In Fig. 5b, cultures induced to express Aβ were co-infected with the combination of adenoviruses expressing Hsp60, -70, and -90 in the presence and absence of quercetin. First, Aβ expression drove down endogenous Hsp60 levels (Fig. 5b, lane 3) as it did in Fig. 5a, lane 3 versus 2. Next, the co-expression of all the heat shock proteins is shown to dramatically reduce Aβ levels (Fig. 5b, lane and bar sets 5 versus 3). This result was not predictable from the data in SY5Y cells in which the HSPs independently had more modest effects on Aβ levels (Fig. 1c). As expected, quercetin did not reduce levels of constitutively driven HSPs (Fig. 5b, lane and bar sets 6 versus 4). However, quercetin did partly overcome the Aβ-reducing effect of the overexpressed panel of HSPs, probably through the reduction in Hsp60 and 70 levels, in the context of cell stress (Fig. 5b, lane 7 versus 5). These data indicate that the combined action of expressed heat shock proteins is to greatly reduce accumulation of intracellular Aβ in cortical neurons, even overcoming to some extent the effect of quercetin to depress levels of both endogenous and expressed Hsp60, including to a lesser extent Hsp70, under Aβ/oxidative stress conditions.

After the demonstration that heat shock protein expression acts to reduce steady-state levels of intracellular Aβ, we determined whether this correlated with a reduction in Aβ mediated cytotoxicity. In Fig. 5c, LDH release into the medium increased >3-fold in Aβ poisoned cultures (Fig. 5c, bar 2 versus 1). Expressed individually, Hsp60 and Hsp70 significantly decreased LDH release in the presence of Aβ induction with doxycycline. Hsp90 overexpression appeared to be less effective in this regard. Combined overexpression of all the three HSPs provided maximum protection against cell death (Fig. 5c, bar 6 versus 2). The addition of quercetin treatment to Aβ-laden cultures led to the highest levels of cytotoxicity (Fig. 5c, bar 7) by presumably acting on endogenous HSP levels (See Fig. 5a, lane 6). However, the combination of overexpressed heat shock proteins neutralized this negative influence on cell survival (Fig. 5c, bar 8 versus 7). This result is in line with the reduction of Aβ levels seen in Fig. 5b, lane 7.

To further differentiate mitochondrial versus Aβ-lowering mechanisms by which the individual HSPs confer cytoprotection, the respiratory activity corresponding to each of the ETC complexes was measured in the presence of Aβ. Intracellular expression of Aβ-(1–42) did not alter oxygen consumption through either complex I or complex II/III respiration (Fig. 6a,
Hsp60 Protects against Aβ-induced ETC Inhibition

**FIGURE 5.**

*a*, Western blot (WB) and the densitometry analysis shows a marked decrease in endogenous Hsp60 but not in Hsp70 or Hsp90 levels in Aβ-stressed cortical neurons (*lane 3*). The addition of 100 μM quercetin profoundly raises levels of expressed-Aβ in the cortical neurons while reducing Hsp70 and eliminating Hsp60 levels (*lane 6 versus 3*). *b*, the accumulation of Aβ is significantly reduced when Hsp60, Hsp70, and Hsp90 are co-expressed as a group (*lane 5, bottom panel*). The addition of quercetin did not affect the Hsp70 and Hsp90 levels driven by virus but is found to have a major reducing effect on Hsp60 (*lane 7*). Quercetin also partially reversed the anti-Aβ property of HSPs. The decreased level of Hsp60 in quercetin-treated (*lane 7*) may be a function of renewed accumulation of β-amyloid or a direct action. The Western blot is representative of three different experiments. *c*, overexpression of Hsp60 and Hsp70 each significantly decrease LDH release after Aβ expression (*bar 3 and 4*). Hsp90 was only partially effective. The combined overexpression of all three HSPs showed maximum abrogation of LDH release, achieving normal levels (*bar 6*). The addition of quercetin magnified Aβ-induced LDH release to maximal levels (*bar 7*). Combined overexpression of Hsp60, Hsp70, and Hsp90 nearly overcame the quercetin/Aβ effect (*bar 8*), n = 3 in each case, p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***). Compared with control, p < 0.05 (*), p < 0.01 (**), and p < 0.001 (***). Compared with β-amyloid stressed group, Quercetin alone was identical to the bar 1 result (not shown). Densitometry of Western blots is normalized to cdk4 and their control. The asterisk in the densitometry of *b* represents the endogenous expression of HSP levels. Error bars ± S.D.
FIGURE 6. a, intracellular β-amyloid did not alter levels of oxygen consumption by either complex I or complex III activities but significantly depleted complex IV (group 3 versus 2 and 1). Cortical neurons driven to overexpress Hsp60 resulted in significant restoration of oxygen consumption at complex IV (bar 5). No additive effect is observed in the combined group of HSPs (bar set 8). The addition of quercetin to the HSP-protected, Aβ-stressed group (bar 9) resulted in a noticeable drop-off in oxygen consumption at complex IV to nearly Aβ alone levels (bar set 3). n = 3 in each case. p < 0.05 (#) and p < 0.01 (##) compared with control; **, p < 0.01 compared with Aβ-stressed group. ¶, p < 0.05 compared with Aβ-stressed group with added with quercetin (bar 4). Error bars ± S.D.

b, in the upper panel and the densitometry analysis to the right, Hsp60 was most effective in reducing cytochrome c release (lane 5 versus 3). Simultaneous expression of all three HSPs entirely abolished this release (lane 8). The addition of quercetin to the Aβ-poisoned cells increased cytochrome c release in "HSP deficient" (lane 4) and reestablished cytochrome c release in HSP-endowed (lane 9) primary neurons. In the lower panel an increase in the level of full-length caspase-9 and of its activated subunits by cleavage is shown to result from intracellular Aβ expression (lane 3 and 4). Hsp70 and Hsp90 overexpression are minimally protective against caspase-9 activation and cleavage, whereas again it is shown that Hsp60-alone (lane 5) is most efficient in mitigating Aβ. HSPs in combination abolished this activation (lane 8). The addition of quercetin re-established the activation of caspase-9. Densitometry is normalized with respect to cdk4 levels and their control. WB, Western blot. c, quercetin aggravates heat shock-induced-cleaved caspase levels (lane 7 versus 3) and blocks the induction of Hsp60.
Hsp60 Protects against Aβ-induced ETC Inhibition

normalized bar sets 3–8 versus 2 and a no-additions control, bar set 1). Importantly however, there was a substantial reduction in complex IV activity (Fig. 6a, bar set 3 versus 1). This effect of Aβ was nearly completely reversed by the directed overexpression of Hsp60 (Fig. 6a, bar set 5) but not with overexpression of either Hsp70 or Hsp90 (Fig. 6a, bar set 6 and 7). The overexpression of all three heat shock proteins did not lead to any additive effect on the restoration of oxygen generation (Fig. 6a, bar 8). The addition of quercetin to Aβ-affected cells was additively toxic to complex IV (Fig. 6a, bar set 4 versus 3) in register with Fig. 5c, bar 7, results. In this assay quercetin near completely neutralized the beneficial effect of Hsp60 to restore levels of oxygen consumption (Fig. 6a, bar set 9 versus 8 and 5). Quercetin alone at this concentration exhibited no intrinsic cell toxicity by any measure.

Cytochrome c release from the mitochondria into the cytosol acts as a trigger in the pathway of caspase-mediated early apoptotic events. We measured the release of cytochrome c to the cytosol after induction of intracellular Aβ expression and then gauged the effects of heat shock protein overexpression on this release. In the same experiments the subsequent cleavage of inactive procaspase-9 to its active form was assessed. Aβ expression resulted in activation of both steps leading to apoptosis Fig. 6b, lane and bar sets 3 and 4 versus 1 and 2). Overexpression of Hsp60 substantially reduced cytochrome c release to the cytosol as compared with the effects of Hsp70 and Hsp90 overexpression alone (Fig. 6b, lane and bar sets 5 versus 6 and 7). Co-overexpression of all three HSPs entirely abolished cytochrome c release and caspase cleavage (Fig. 6b, lane and bar set 8). The addition of quercetin to the Aβ- and HSP-expressing cells is shown again to neutralize the anti-apoptotic action of HSPs, supporting the hypothesis that functional inhibition of HSPs with emphasis on Hsp60 leads to intracellular Aβ-mediated mitochondrial damage. To confirm the inhibitory activity of quercetin, we added quercetin to the heat shock protocol in SH-SY5Y cells. Quercetin resulted in the enhanced accumulation of cleaved caspase levels in cells unprotected from heat shock-induced apoptosis (Fig. 6c, lane 7 versus 3).

DISCUSSION

Mitochondrial dysfunction is observed in AD, and defects in energy metabolism are a consistent feature of AD-affected brain (31). The activity of several different mitochondrial enzymes appears reduced; the best studied and perhaps most important of these enzyme defects involves cytochrome oxidase (32, 33). Because oxygen radicals are a byproduct of mitochondrial respiration and mitochondrial dysfunction can cause an increase in their production, the mitochondria are likely to be an important source of increased oxidative stress in AD. However, it is not clear whether and to what extent ROS generated from other cell organelles or structures similarly affected by toxic misfolded proteins may first impair mitochondrial function or if pathological changes in them follow from it. Moreover, peroxidation of the very same implicated proteins (e.g. β-amyloid in AD, α-synuclein in PD, and SOD1 in amyotrophic lateral sclerosis (ALS) may have in common an increase in protein misfolding, impaired degradation, and accumulation of toxic soluble protofibrils (3) that may perpetuate a cycle of degeneration. In addition, recent studies have pointed to energy failure as a contributor to neurodegenerative disorders and mitochondrial damage is a leading suspect (34–39).

In the present study we utilized an inducible, adenovirus-based expression vector to raise the levels of intracellular Aβ and have uncovered a specific impairment in the function of complex IV of the ETC. The inhibitory effect of intracellular Aβ is likely to be exerted through a direct or at least via a close intermediate interaction with the protein subunits of complex IV. This view is supported by an increase in the apparent $K_m$ of cytochrome oxidase for its substrate-reduced cytochrome c that is related to Aβ production. The ~50% inhibition of complex IV is the likely cause for the similar reduction in ATP generation we observe. What is not clear but is of central importance is whether the inhibition of the respiratory chain and depletion of ATP results in oxidative stress through the production of reactive oxygen species from electron leak at complexes III and I or whether ROS are formed first and then damage complex IV. Other data from our laboratory on this point show that the addition of vitamins C and E effectively scavenged intracellular β-amyloid-mediated ROS generation but was less effective in protecting the activity of oxidative phosphorylation enzymes and ATP generation (40). These results are concordant with the findings of Casley et al. (41) who show that enzyme complex IV was inhibited by synthetic Aβ in the absence of biological membranes, a major source of ROS generation (33). These data together with our data argue that intracellular Aβ directly interferes with oxidative phosphorylation resulting in oxidative stress rather than indirectly such as first through the generation of free radicals. The current study also gives evidence that follows ROS generation consequent to mitochondrial damage by dissecting the actions of molecular chaperones.

A deficiency in the induction of HSPs is expected to result in a build-up of oxidatively damaged proteins that are resistant to ubiquitinylation and degradation by the 26 S proteasome (42). For instance, depletion of soluble HSPs has been proposed to be a major factor in the cellular degeneration associated with ALS (43). Aside from their contributions to protein folding, refolding, transportation, and translocation, molecular chaperones also induce “thermotolerance” to stresses other than heat shock, including to reactive oxygen species (18, 20). As an example, the heat shock response has been implicated in the prevention of H$_2$O$_2$-induced cell death through an increase in the activity or expression of endogenous free radical scavengers, catalase, and superoxide dismutase (44).

Various mitochondrial-based mechanisms have also been proposed to account for the cytoprotective effects of HSPs. General protection at the mitochondrial level has been demonstrated in cardiac myocytes by Hsp60 (45). In the present study, steady-state accumulation of intracellular β-amyloid was not altered by the overexpression of Hsp60 in neuroblastoma cells, whereas moderate to slight reductions were observed after the overexpression of Hsp70 and Hsp90, respectively. In primary neurons, a dramatic reduction in Aβ was obtained by the combined action of all HSPs. This synergistic effect will require more experimentation to explain in greater detail, since Hsp70 alone has no effect on Aβ levels per se in primary neurons (22).
One plausible explanation for these observations is that some HSPs (e.g. 70 and 90) may act to attenuate the cytotoxicity of Aβ by preventing processes such as oxidation, oligomerization, misfolding, and aggregation of Aβ in some instances and assisting degradation of Aβ in others. From our study these would not necessarily involve mitochondrial mechanisms. Other HSPs such as mitochondrial chaperones 60/10, although susceptible to Aβ in their abundance, do not in turn affect Aβ levels or oligomerization but do protect vulnerable components of key organelles such as complex IV of the ETC and some components of the mitochondrial matrix. Of particular interest, accumulation of intracellular β-amyloid consistently depleted endogenous Hsp60 levels in primary neuronal cultures without similarly effecting Hsp70 and Hsp90 levels. Previous reports had identified a similar reduction in Hsp60 upon synthetic β-amyloid treatment in cortical neuron cultures (46). To our knowledge, no previous studies have examined the role of mitochondrial Hsp60 either alone or in combination with the other two major heat shock proteins, Hsp70 and Hsp90, on integrated mitochondrial dysfunction induced by either intracellular or extracellular β-amyloid. Our results demonstrate that expression of Hsp60 alone and additively in combination with Hsp70 and 90 preserved oxidative phosphorylation (ATP production) and cell viability (LDH release) through the protection of respiratory complex IV. We found that Hsp60 in particular maintained the apparent Km for cytochrome oxidase in neuroblastoma cell stressed by the accumulation of intracellular β-amyloid. These results are in agreement with earlier reports where overexpression of Hsp60 in myocytes improved the function of individual ETC complexes, III and IV in particular, when they were subjected to ischemia and reoxygenation (45).

Neurons require a constant glucose supply for the generation of ATP, and mitochondrial dysfunction in AD includes the diminished activities of ATP regulating enzymes, creatine kinase, PDH, and α-KGDH (47–49). The mechanism of their inhibition may proceed through intracellular β-amyloid-mediated free radical generation and indirect oxidative damage or, alternatively, through direct interaction with Aβ (1–42). However, only partial protection of the enzyme complexes PDH and α-KGDH from intracellular β-amyloid was obtained from the addition of antioxidants, suggesting again the possibility of a more direct form of inhibition by β-amyloid, which has no absolute requirement for ROS intermediary (40). On the other hand, Hsp60 and Hsp90 overexpression alone was each sufficient to prevent PDH and α-KGDH inactivation, respectively, and in combination with Hsp70 provided a slight additive effect. Yet it is still possible that protection of PDH activity may benefit through decreases in ROS production, since in our study the overexpression of Hsp60 correlated best with a decrease in ROS generation.

In support of the notion that HSPs in concert serve to reduce Aβ- and Aβ-induced cytotoxicity, the addition of quercetin to primary cortical neurons led to increased accumulation of intracellular Aβ and exaggerated the Aβ-induced cell death (Fig. 5c), mitochondrial complex IV activity (Fig. 6a), and apoptosis (Fig. 6b). Although quercetin alone did not affect resting endogenous or overexpressed HSP levels (Fig. 5, a and b), it significantly reduced endogenous and expressed Hsp60 (and -70) levels under conditions of Aβ cellular stress. The depletion of HSP reserves and function (e.g. Fig. 6c) we observe is consistent with these mechanisms of quercetin action (29, 30, 50).

Another mechanism for the identified protective effect of HSPs is likely related to their ability to directly interfere with mitochondrial apoptotic pathways. The mitochondrial pathway is initiated by the release into the cytosol of cytochrome c and other soluble apoptogenic molecules that include apoptosis inducing factor, Smac/Diablo, Htra2/Omi, and endonuclease G. Cytochrome c, once in the cytosol, interacts with apoptotic protease activation factor–1 (Apaf-1), thereby triggering the ATP-dependent oligomerization of Apaf-1. Oligomerized Apaf-1 then binds to procaspase-9, leading to the formation of the apoptosome, the caspase-9 activation complex (51). Activated caspase-9 triggers the proteolytic maturation of procaspase-3, setting in motion the caspase activation cascade responsible for apoptotic cell death (52). In the present study we have found that increased expression of Hsp60, Hsp70, and Hsp90 individually and especially in ensemble, reduce cytochrome c release and activation of caspase-9 by β-amyloid accumulation at several stages of the apoptotic pathway. Hsp60 is especially adept at protecting complex IV and PDH damage from intracellular Aβ and defending ATP levels. Thus, mitochondrial HSP may qualify as a useful therapeutic target to develop in early AD where intracellular Aβ is a pathogenic factor.

In summary, we have found that the accumulation of intracellular Aβ decreases the activity of important components of the cellular energy generation system, compromises mitochondrial function, and leads to cell death. Overexpression of Hsp60 alone and in combination with Hsp70 and Hsp90 protected neurons by reducing the cytotoxicity induced by intracellular β-amyloid. Preservation of the ETC, maintenance of ATP generation, decreased ROS generation, decreased cytochrome c release, and lowered caspase-9 activation by the molecular chaperones identifies their restorative roles as well as processes compromised by Aβ. Inhibition of the heat shock proteins using quercetin confirmed the role of molecular chaperones to protect the neuron against intracellular β-amyloid induced dysfunction.

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Hsp60 Protects against Aβ-induced ETC Inhibition
