Mitochondrial aldehyde dehydrogenase-2 activation prevents β amyloids induced endothelial cell dysfunction and restores angiogenesis

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Summary

Amyloid β peptides (Aβ1-40 and Aβ1-42) cause cerebral degeneration also by exerting antiangiogenic properties, as impairment of angiogenic factors activity and induction of apoptosis and senescence in the endothelium. Amyloid peptides are known to induce oxidative stress. Impairment of mitochondrial aldehyde dehydrogenase 2 (ALDH2) following oxidative stress, results in accumulation of toxic aldehydes, particularly 4-hydroxynoneal (4-HNE). We sought to determine the role of mitochondrial ALDH2 in Aβ-related impairment of angiogenesis. We hypothesized that by increasing the detoxification activity of ALDH2, we would reduce Aβ-driven endothelial injuries and restore angiogenesis. We used a selective ALDH2 activator, Alda-1, assessing its ability to repair mitochondrial dysfunction in the endothelium. Treatment of human endothelial cells with Aβ1-40 (5-50 μM), induced loss of mitochondrial membrane potential, increased cytochrome c release and ROS accumulation. These events were associated with 4-HNE accumulation and decrease in ALDH2 activity (40%), and resulted in disassembly of endothelial junctions, as evidenced by β-catenin phosphorylation, disorganization of adherens and tight junctions, and by disruption of pseudocapillary formation. Alda-1 (10-40 μM) abolished Aβ-induced 4-HNE accumulation, apoptosis, and vascular leakiness, fully restoring the pro-angiogenic endothelial phenotype and responses to FGF-2. Our data document that mitochondrial ALDH2 in endothelium is a target for the vascular effect of Aβ including loss of barrier function and angiogenesis. ALDH2 activation, by restoring mitochondrial functions in endothelium, prevents Aβ-induced dysfunction and antiangiogenic effects. Thus, agents activating ALDH2 may reduce endothelial injuries including those occurring in cerebral amyloid angiopathy, preserving the angiogenic potential of the endothelium.
Introduction

A wealth of evidence indicates the mitochondria dysfunction is a feature common to many age-related degenerative pathologies, including β amyloid-driven diseases such as Alzheimer’s disease (AD) and cerebral amyloid angiopathy (CAA) (Lin and Beal, 2006, Cho et al., 2009). β amyloids have been shown to localize mainly at the inner mitochondrial membrane, causing excessive reactive oxygen species (ROS) production (Lin and Beal, 2006). Besides the respiratory chain injuries, β amyloids have been shown to enhance the expression of cyclophilin D, a mitochondrial protein regulating the opening of the transition pore (Du et al., 2008), and to promote S-nitrosylation of dynamin-related protein 1, a protein that governs mitochondrial dynamics (Cho et al., 2009). β amyloids also form a complex with the mitochondrial Aβ-peptide-binding alcohol dehydrogenase (ABAD), an interaction which leads to accelerated cell death (Yao et al., 2011).

In addition, β amyloid-induced ROS elevation activates lipid peroxidation pathways, a mechanism that generates excessive amounts of highly toxic aldehydes, particularly 4-hydroxy-2-noneal (4-HNE) (Schneider et al., 2008). Because of its electrophilic nature, 4-HNE forms stable adducts with a myriad of proteins required for mitochondrial functions (Petersen and Door, 2004). 4-HNE exerts toxic effects in a wide range of tissues, including neural and cardiac tissues (Neely et al., 1999; Che-Hong et al., 2008) and endothelium (Suzuki et al., 2007).

Although metabolized by a number of detoxifying mechanisms, 4-HNE is a specific substrate of the mitochondrial aldehyde dehydrogenase 2 (ALDH2) (Che-Hong et al., 2008; Hyun et al., 2002; Mattson, 2009). In a rat myocardial infarction model, boosting the ALDH2 activity, through a novel enzyme activator, Alda-1, resulted in 60% decreased injury from the ischemic damage, an effect associated with enhanced clearing of the toxic 4-HNE (Che-Hong et al., 2008).

β amyloid peptides, particularly the shorter vasculotropic Aβ1-40 (hereafter termed Aβ), affect brain blood vessels, altering important functions of medium and small arterioles and capillary endothelium. These derangements include impairment of vasoactive tone, vascular remodeling, and barrier functions, as well as suppression of the intrinsic angiogenic properties of endothelium, such as responsiveness to fibroblast growth factor-2 (FGF-2), FGF-2 expression, its cognate receptor FGFR1, and, also, vascular endothelial growth factor receptor2 (VEGFR2) (Revesz et al., 2003; Patel et al., 2010; Solito et al., 2009). Collectively, these alterations contribute to CAA, a pathology characterized by Aβ1-40 deposition around cortical and leptomeningeal vessels, frequently occurring in AD patients (>80%).
Investigations from various laboratories have delineated a range of Aβ-induced biochemical and signal changes that underlie the disease. The observation that Aβ strongly activates the intrinsic apoptotic pathway is perhaps the clearest evidence that these peptides target the mitochondria to execute their toxic effects (Fossati et al., 2010).

We sought to determine the role of mitochondrial ALDH2 in Aβ related impairment of endothelial functions and FGF-2-mediated angiogenesis. To investigate ALDH2 in angiogenic events, and in amyloid angiopathy, we used the recent identification of a novel class of small molecular weight compounds that selectively interact with the mitochondrial enzyme increasing its catalytic activity. Specifically, Alda-1 (benzodioxyl dichlororobezamide), the prototype of compounds that activate ALDH2, inhibits the injuries produced by 4-HNE in several model of oxidative stress (Che-Hong et al., 2008; Perez-Miller et al., 2010).

The use of this pharmacological tool enabled to delineate the role of mitochondrial ALDH2 in Aβ-impaired angiogenesis.
Results

Aβ treatment causes mitochondrial impairment in cultured endothelial cells

The effects induced by Aβ on the endothelium have been described in numerous reports (Revesz et al., 2003; Patel et al., 2010; Solito et al., 2009). Here we focused on the Aβ-induced mitochondrial dysfunction in endothelial cells, human umbilical endothelial cells (HUVEC) and human brain microvascular endothelial cells (HBMEC). To characterize the mitochondrial damage induced by Aβ, we evaluated a number of mitochondrial functions. Aβ produced a concentration-dependent loss of mitochondrial membrane potential ($\Delta \psi_m$), as measured by JC-1 monomer levels (Fig. 1A). The reverse peptide (Aβ$_{40-1}$), since did not affect mitochondrial potential relative to no peptide addition (5756 ± 429 vs. 5880 ± 307, fluorescence levels respectively), was used as control throughout the manuscript. There was also a 7 folds increase in cytochrome c release in Aβ treated HUVEC (Fig. 1B). Impairment of mitochondrial potential affected the integrity of the electron transport system, producing a marked rise of super oxide formation, detected by MitoSox (Fig. 1C). A similar loss of $\Delta \psi_m$, as well as cytochrome c release and ROS overproduction occurred in HBMEC exposed to Aβ (Fig. 1D,E,F).

To investigate the molecular mechanism of Aβ-induced mitochondrial dysfunction, we determined whether the lipid-peroxidation generated aldehyde, 4-HNE, accumulates following Aβ treatment. In particular, as this aldehyde is very reactive and forms adducts on macromolecules, including proteins (Petersen and Door, 2004), we measured 4-HNE protein adducts detected by ELISA (Fig. 2A) and immunohistochemistry (Fig. 2B) in Aβ-treated cells. There was a three-fold increase in 4-HNE adduct formation (Fig. 2A and 2B, panel b), especially around the perinuclear area (Fig. 2B, panel b). To investigate whether 4-HNE adducts localized in mitochondria, we performed the double staining of 4-HNE adduct formation in combination with an antibody against the TOM 20 (a traslocase of the outer mitochondrial membrane; Fig. 2C, panel a). We detected a co-localization of 4-HNE within the mitochondria (Fig. 2C, panel b), along with partial overlap of the two markers in the organelles. Since ALDH2 is the main enzyme that metabolizes 4-HNE, we determined whether selective activation of ALDH2 with Alda-1 would decrease the levels of 4-HNE adducts. Co-treatment of HUVEC with Alda-1 and Aβ caused more than 60% decrease in 4-
HNE adduct accumulation (Fig. 2A), drastically reducing the 4-HNE associated immunofluorescence (Fig. 2B and C; compare panels c vs. b in both figures).

To determine whether Aβ-induced mitochondrial dysfunction is caused by adduct accumulation, we determined the benefit of Alda-1 (10-40 µM) on mitochondrial membrane potential. The two highest Alda-1 concentrations provided comparable protection of mitochondrial potential, whereas the lower one was ineffective (Fig. 3A). All further experiments of this study were conducted with 20 µM of Alda-1. Confocal microscopy performed in HUVEC with the JC-1 stain demonstrated a clear difference between mitochondrial membrane potential ($\Delta \psi_m$) in Aβ-treated cells versus those pretreated with Alda-1. The diffused red fluorescence in control cells denotes the presence of JC-1 aggregates typical of high $\Delta \psi_m$ (Fig. 3B, upper panels), whereas the abundant green fluorescence in Aβ-treated cells indicates low $\Delta \psi_m$, typical of JC-1 monomers (Fig. 3B, middle panels). Note the partial reversal of Aβ-induced decline in mitochondrial potential by Alda-1, as evidenced by the coexistence of red and green fluorescence in the merge images (Fig. 3B, bottom panels). Mitochondrial associated damage was also observed by measuring cytochrome c levels and apoptosis evaluated by phosphatidylserine immunostaining. Alda-1 treatment abolished Aβ-induced changes in the above mentioned parameters (Fig. 1B, E, and Fig. 3C). Together these data support our hypothesis that Aβ causes mitochondrial dysfunction by inducing toxic 4-HNE accumulation, and that the resulting injuries can be counteracted by stimulating ALDH2 activity through a specific enzyme activator.

Aβ reduces ALDH2 enzymatic activity

In view of the marked 4-HNE increase in endothelial cells following Aβ exposure, we measured ALDH2 activity (Che-Hong et al., 2008). Application of 50 µM Aβ to HUVEC substantially reduced ALDH2 activity by 40% (Fig. 4A), exerting negligible effects on its expression (Fig. 4B). This reflects an ALDH2 inactivation by 4-HNE (Che-Hong et al., 2008), rather than a direct interaction of Aβ with the enzyme. In fact, incubation of Aβ (10-50 µM) with recombinant ALDH2, did not reduce its enzymatic activity (Fig. 4C). As reported (Che-Hong et al., 2008), addition of Alda-1 to the recombinant enzyme produced a significant increase of its catalytic activity (Fig. 4C). Importantly, administration of Alda-1 prior Aβ treatment prevented the decline in ALDH2 activity in both HUVEC (Fig. 4E, p<0.01) and in HBMEC (Fig. 4F, p< 0.05). Further, the marked expression of ALDH2
compared to that of ALDH1 in HUVEC, suggests its preeminent involvement in the removal of 4-HNE in endothelial cells (Fig. 4D). To examine the possibility that reactive species other than 4-HNE were involved in ALDH2 decreased activity, we used optimal concentration of MnTBAP, a cell permeable superoxide dismutase (SOD) mimetic (Cantara et al., 2007), and measured ALDH2 activity in HUVEC previously exposed to Aβ. The scavenger was effective in reversing Aβ-decrease ALDH2 activity, although its efficacy was lower compared to that of Alda-1 (Fig. 4E, p>0.05 vs. p>0.001).

**ALDH2 activation prevents endothelial cell membrane disorganization and permeability caused by Aβ**

We investigated whether Aβ would affect the adherens and tight junctions of endothelial cells causing their functional impairment. To this end, we examined signaling molecules, i.e., VE cadherin, β-catenin and ZO-1 protein, all known to be involved in maintaining adherens and tight junction integrity. Aβ induced cytoplasmic β-catenin phosphorylation (Fig 5A), an event known to provoke its dissociation from VE-cadherin, thus leading to disassembly of adherens junction (Chen et al., 2012). Indeed, Aβ also abolished the distribution of VE cadherin at cell-cell contact, evaluated by immunofluorescence (Fig. 5B, panel b vs a). Moreover, since adherens junctions influence tight junctions organization, (Dejana, 2004) we investigated, by immunohistochemistry, the expression pattern of the tight junction protein, ZO-1. Aβ also abolished the typical distribution of ZO-1 lining the plasma membrane at the cell-cell contacts (Fig 5B, panel e vs d). Treatment with Alda-1, prevented all the above changes, hence preserving the integrity of endothelial junctions (Fig. 5A and B panel c vs. b and panel f vs. e). Taken together, these evidences converge in indicating that activation of ALDH2 contributes to maintain the integrity of endothelial cell junctions, conceivably by shielding them from the toxic insults of 4-HNE.

In line with the above findings, we observed a change of permeability in HUVEC following Aβ exposure, as shown by the large paracellular flux increase of fluorescent-conjugated dextran, which exceeded by 30% that recorded in control. In contrast, Alda-1-pretreated cells exhibited a paracellular flux comparable to control. (Fig. 5C).
Angiogenic phenotype and angiogenesis response disrupted by Aβ are restored by Alda-1

Beta amyloids severely impair angiogenic responses of the endothelium, as previously reported (Donnini et al., 2010, Paris et al., 2005). Here, we show that HUVEC, seeded on matrigel, produce an organized pseudocapillary network (Fig. 6A, panel a, and insert i), while they fail to organize a proper architecture in the presence of Aβ (Fig. 6A, panel b). The observed cell clustering and stunted growth, occurring in the presence of Aβ, was likely due to impaired cell adhesion and migration (Fig. 6A, insert ii). Of note, only a few endothelial cells enter apoptosis as documented by phosphatidylserine immunofluorescence (Fig. 3C) and trypan blue staining (data not shown). Experiments on cell migration showed a significantly reduced migration (40%, p< 0.01) caused by Aβ (Fig. 7A). The severe endothelial injuries observed were invariably counteracted by prior application of Alda-1. In fact, the pseudocapillary architecture and cell migration were fully maintained in presence of Alda-1 (Fig. 6A, panel c, and 7A) as also shown in the graph representing quantification of pseudocapillaries (Fig. 6B).

In the context of neovessel formation, we and others have reported that Aβ reduces the expression of major endothelial growth factors, e.g. FGF-2 and VEGF, as well their capacity to elicit angiogenic responses (Donnini et al., 2010 Solito 2009, Paris et al., 2005). We therefore sought to learn whether ALDH2 activation would prevent these Aβ induced injuries. Indeed, Aβ reduced endogenous FGF-2 expression in HUVEC by approximately 50%, an effect completely prevented by the activation of ALDH2 via Alda-1 (Fig. 7B). Also, Alda-1 restored responsiveness to exogenous FGF-2 (20 ng/ml) in the formation of the network of pseudocapillaries, impaired by Aβ, as documented by the quantification of pseudocapillary network formation (Fig. 6A, panels d-f, Fig. 6B). Similarly, Alda-1 recovered the capability of endothelial cells to respond to FGF-2, when HUVEC where embedded in the fibrin gel and exposed to Aβ, restoring their angiogenic phenotype (Fig. 6C and D).
The mitochondrial enzyme aldehyde dehydrogenase 2 (ALDH2) exerts an important physiological protection in a variety of tissues, as it degrades highly toxic aldehydes originating from the lipid peroxidation cascade, mainly the aldehyde 4-hydroxy-2-noneal (4-HNE). Protection afforded by ALDH2 has been observed in conditions in which oxidative stress and the resulting excessive production of 4-HNE causes tissue damage, often evolving toward degenerative processes (Ohsawa et al., 2008; Wey et al., 2012). Thus, ALDH2 appears to be implicated in diverse pathologies such as Alzheimer and Parkinson diseases and ischemic conditions (Ohsawa et al., 2008; Wey et al., 2012). Here, we examined the role of ALDH2 in the development of endothelial dysfunction produced by the vasculotropic amyloid Aβ (Aβ1-40), known to be involved in the pathogenesis of the Alzheimer-associated cerebral amyloid angiopathy (CAA) (Donnini et al., 2010).

Acute Aβ exposure of endothelial cells, HUVEC and HBMEC, representing peripheral and CNS vascular districts, produced a marked mitochondrial dysfunction as shown by the dramatic loss of mitochondrial membrane potential and by the rise of cytochrome c release. Mitochondrial injuries occurred concomitantly to the surge of superoxide and to 4-HNE adducts accumulation. These changes occurred rapidly, within 30 minutes, and therefore likely to be caused by short oligomers of Aβ (Solito et al., 2009). A number of studies have illustrated a direct interaction between Aβ and alcohol dehydrogenase (ABAD) in neurons, which prevents NAD+ binding to the active site in ABAD and results in oxidative stress (Yao et al., 2011). In contrast, such direct interaction of Aβ with ALDH2 does not occur (Fig. 4C). Therefore, the sharp decline of ALDH2 enzymatic activity, noted in endothelium following Aβ treatment, is clearly related to the oxidative injuries to the mitochondria, rather than a direct effect of the Aβ on ALDH2.

This is the first study that shows the decline of ALDH2 activity in endothelial cells treated with Aβ, pointing to ALDH2 activity decline as the mechanism of Aβ toxicity. Indeed, by using a specific activator of ALDH2, termed Alda-1, we found that the ALDH2 recovery fully restored mitochondrial functions, in terms of membrane potential and cytochrome c release. Alda-1 treatment reduced 4-HNE adducts and ROS formation, and rescued Aβ-impaired functions of the endothelium, supporting the hypothesis that impaired detoxification of biogenic aldehydes may be important in Aβ-induced vessel injuries. The molecular mechanism whereby Alda-1 protects ALDH2, described in a report from one of our laboratories, illustrates how the compound reduces the accessibility of 4-HNE to key cysteine
residues of the enzyme, essentially by prolonging the residence of NAD, the enzyme cofactor (Perez-Miller et al., 2010).

The activation of ALDH2, through Alda-1, was a remarkably efficient mean to contrast the Aβ-induced injuries on the endothelium. In fact, protection was evident on both basic and FGF-2-mediated endothelial functions, such as the ability of cultured endothelium to express an angiogenic phenotype (pseudocapillary formation, and FGF-2 production) and to maintain the barrier function. These are important determinants of endothelial viability, as they are the basis of vessel remodeling and protection of the underlying tissues from toxic insults.

Further, since FGF-2 is an important prosurvival and pro-angiogenic factor, the responsiveness to FGF-2, promoted by ALDH2 activation, is a relevant determinant of endothelial angiogenic potential.

Analysis of signals involved in endothelial barrier revealed an increase phosphorylation of β-catenin, disorganization of the tight junction ZO-1 protein, and of adherens junction VE cadherin in cells exposed to Aβ, indicative of intercellular gap opening, and increased permeability (Murakami and Simons, 2009; Chen et al., 2012). The neurovascular unit in the brain is a complex comprising endothelial cells, neurons, pericytes and astrocytes (Wey et al., 2012). Because components of the unit are tightly interconnected, it is plausible that damage of one component may reflect on the functioning of other cells. Perturbations of endothelial barrier functions may expose all components to toxic insults (e.g. amyloids) and, in addition, may impair their disposal through the well documented mechanisms (Quaegebeur et al., 2011; Zlokovic et al., 2008). Conceivably, the observed reversal by Alda-1 of enhanced permeability may be crucial for restoring the integrity of the blood brain barrier.

The involvement of mitochondria in vascular lesions, caused by Aβ peptides or other insults inducing ROS, remains largely undefined in terms of molecular mechanism. An earlier work reported histological evidence for vessel injuries associated with the mitochondrial dysfunction found in AD patients specimens, and in a mouse model of AD (Aliev et al., 2003). More recently, reports have described the protection of cerebral vasculature morphology and function in transgenic mice lacking the ROS-producing machinery through ablation of Nox-2 gene (Park et al., 2004). Mitochondrial involvement in vessel injuries has been demonstrated in reports showing that TAT-linked BH4, a peptide domain of the antiapoptotic Bcl-xL mitochondrial protein, prevented Aβ-induced damages in cultured endothelial cells, and ischemic brain damage (Donnini et al., 2009, Cantara et al., 2007).

Recently, a mitochondrial protein, termed prohibitin-1, has been shown to protect the endothelium from ROS damages (Schleicher et al., 2007). Together, these findings
underscore the relevance of targeting lipid peroxidation products as a means of rescuing vessels from toxic insults.

The present work identifies a specific detoxifying mechanism, provided by activation of a mitochondrial enzyme, ALDH2, by means of the selective activator Alda-1. The protection of this activator toward the vascular endothelium from injuries arising from lipid peroxidation toxic products, may have implication for a wide range of diseases in which impairment mitochondrial function and angiogenesis are the underlying pathogenetic mechanism. Our findings, demostrating that activation of ALDH2 by Alda-1, prevents the injurious effects of Aβ on vascular endothelium and preserves angiogenic phenotype and responsiveness, may have applications for other age-related vasculopathies, including those associated with diabetes complications and vascular dementia.
Materials and Methods

Reagents

Aβ1-40 and its reversed sequence (control), synthesized at Espikem (University of Florence), were dissolved as previously described (Solito et al., 2009; ). MitoSox™ Red mitochondrial superoxide indicator (M36008) was from Molecular Probes, Invitrogen, (Paisley PA4 9RF, UK). JC-1, mitochondrial membrane potential probe (N.10009142) was from Cayman Chemical Company (Ann Arbor, Michigan 48108, USA). Oxiselect™ HNE–His Adduct ELISA Kit (No.STA-334) was from Cell Biolabs Inc.(7758 Arjons Drive, San Diego, CA 92126 USA). Acetaldheyde was from Sigma, (St. Louis, MO, USA). MnTBAP was from Cayman Chemical Company (Ann Arbor, Michigan 48108, USA).

Cell Culture

Human umbelical vascular endortelial cells (HUVEC, Cambrex, East Rutherford, NJ, 07073 USA), and human brain microvascular endothelial cells (HBMEC, CellScience, Canton, MA, 02021, USA) were cultured as reported (Solito et al., 2009; Donnini et al., 2010).

Detecting mitochondrial membrane potential (ΔΨm)

Changes in ΔΨm were evaluated using JC-1, a dye that selectively enters into mitochondria and changes color from green to red as the potential increases. 2 x 10^4 cells/well/100 μl cells were seeded into a 96-well plate, pretreated with Alda-1 (20 μM, 30 min) then exposed to Aβ (5, 25, 50 μM, 30 min). After treatments, JC-1 staining solution was added to each well (20 min), then cells were washed. Changes in ΔΨm, detectable by the presence of different JC-1 forms (either green or red fluorescence) were quantified by a fluorescence plate reader. JC1 aggregates produce a strong red fluorescence with excitation/emission at 560/595, respectively. JC-1 in the monomeric form produces mostly green fluorescence at excitation/emission wavelength of 485/535 nm, respectively. The presence of JC-1-aggregates and/or the monomeric forms was evaluated by confocal microscopy on cells seeded on glass coverslips in a 24-well culture plate.

MitoSOX Red mitochondrial superoxide indicator

Cells (2.5 x 10^5) were seeded in a 24-well culture plate and treated as above. After treatments, MitoSOX reagent solution (5 μM) was added for 10 minutes. Fluorescence at
excitation/emission wavelength of 510/580 nm, respectively, was observed by an inverted fluorescence microscope (Eclipse TE 300, Nikon).

**OxiSelect HNE-His Adduct Elisa kit**

Cells (3 x 10^5 cells) seeded in 6 cm diameter Petri dishes were treated as above, then scraped in 200 μl lysis buffer (50 mM Tris HCl, 50 mM NaCl, 1 mM EGTA, without Triton X100 or other detergents), sonicated, and centrifuged at 12000 rpm for 20 min at 4 °C. Supernatants (100 μl, 10 μg/ml protein) were adsorbed onto a 96-well plate for 2 hours at 37 °C. The 4-HNE-protein adducts content, probed with an anti HNE-His antibody, was measured by an HRP-conjugated secondary antibody, and quantified by comparison with a 4-HNE-BSA standard curve.

**Aldehyde dehydrogenase (ALDH) enzymatic activity**

ALDH enzyme activity was determined by measuring the conversion of acetaldehyde to acetic acid, as reported (Che-Hong et al., 2008). Briefly, cells were cultured as above, then scraped in 600 μl lysis buffer (100 mM TrisHCl pH 8.0, 10 mM DTT, 20% glycerol, 1% Triton X100), and centrifuged at 13000 g for 20 min at 4°C. The supernatant was used to detect ALDH activity at 25 °C by monitoring NADH formation from NAD^+, at 340 nm in a spectrophotometer (BioRad, Hercules, CA 94547, USA). The assay mixture (0.8 mL) contained 100 mM sodium pyrophosphate pH 9.0, 10 mM NAD^+, and 600 μg of sample protein. The reaction was started by adding acetaldehyde (10 mM) to the cuvette. Enzyme-specific activity was expressed as nmol NADH/min/mg protein. The effect of Aβ on purified human recombinant ALDH2 activity was determined as reported (Che-Hong et al., 2008). Briefly, Aβ (up to 50 μM), DMSO, or Alda 1 or MnTBAP were co-incubated with the enzyme and its activity was determined as described above.

**Western Blotting**

Cells were scraped in a lysis buffer containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 10 mM NaF, 1% Triton and 1% protease inhibitor cocktail. Equal amounts (60 μg) of protein were separated by SDS-PAGE onto a 10% gel and transferred to a nitrocellulose membrane. The membranes were blocked (1 hour) in a solution of 5% (wt/vol) milk and then incubated overnight at 4°C with the primary antibodies (dil 1:1000): anti cytochrome c (Cell Signaling, Millipore, Billerica, MA, USA) or anti ALDH2 (SIGMA) or anti ALDH1 (EPITOMICS, Burlingame, CA 94010-1303 USA) or anti pFAK (Tyr 861)
In vitro angiogenesis model

Cells, pre-treated with Alda-1 (20 µM for 30 min) were exposed to Aβ (25 µM). Cells were then plated onto a thin layer (300 µL) of basement membrane matrix (Matrigel growth factor reduced; Becton Dickinson, Waltham, MA, USA) in 24-well plates at 6 x 10⁴ cells/well in EBM (EGM-2 containing 2.5% FBS) and incubated at 37°C in 5% CO₂ for up 18 hours. Quantification of tubular structures and photomicrographs were performed as previously described (Donnini et al., 2010).

Microcarrier cell culture

Gelatine-coated cytodex microcarriers (MCs) (Sigma-Aldrich, Italy) were prepared and embedded in a fibrin gel as described (Solito et al., 2009). Cells were stimulated with FGF-2 (20 ng/ml,) either alone or together with Aβ (25 µM) and Alda-1 (20 µM). After 2 days the area occupied by capillary-like formations was evaluated in an inverted microscope (20x magnification), using an ocular grid. The sprouting area was expressed as the number of grid units required to cover the entire pseudo-capillary surface. Results are reported as mean area units ± SEM.

Cell migration

Cell migration assay was performed in a 48-well Boyden-chemotaxis chamber (Neuroprobe Inc, Gaithersburg, USA). Briefly, trypsinized HUVEC were resuspended in culture medium with 0.1% FCS, containing or not Alda-1 (20 µM, 30 minutes) and further incubated with Aβ (25 µM, 30 min). Cells (12.5 x 10⁴) suspended in 50 µL medium were loaded into the upper compartment and were incubated for 4 hours at 37 °C with 5% CO₂. Migration was evaluated as number of cells migrated/well.

Immunohistochemistry

Cells were cultured on coverslips, treated with Aβ 50 µM in presence/absence of Alda-1, and then fixed in paraformaldheyde (4%, 5 min), washed in PBS, and incubated with BSA (45
Cells were then incubated for 16 hours with an anti-TOM20 (Sigma), or anti 4-HNE (Santa Cruz), or phosphatidylserine (Upstate), or anti-ZO-1 (BD Transduction, Millipore, Billerica, MA, USA) or anti-VE cadherin (EBioscience, San Diego, CA, USA), diluted 1:50 in PBS containing 0.5% BSA. After incubation with the secondary antibody anti Rabbit IgG TRITC or anti Mouse IgG FITC (1 hour), cells were washed and the coverslips mounted in Mowioll @4-88.

**Permeability**

Cells were seeded on collagen-coated insert membranes (Corning, Tewksbury, MA 01876, USA) containing a high density 0.4 µm diameter pores, and the inserts were placed in a 12 multiwell plate. Cells were seeded at 8 x 10⁴/insert and cultured for 72 hours. Monolayers were treated with Aβ (50 µM, 24 hours) with or without Alda-1 (20 µM), then a 3 kDa FITC-Dextran (10 µM) diluted in PBS, was added on top of cells, allowing the fluorescent molecules to pass through the endothelial cell monolayer. The extent of permeability was determined in a time range (0-60 min) by measuring the fluorescence in a plate reader (Tecan), at 485/535 nm, excitation/emission, respectively. Arbitrary values were plotted against time.

**Statistical analysis**

Results are expressed as means ± s.e.m. Statistical analysis was performed using Student’s t test, or analysis of variance (ANOVA). P<0.05 was considered statistically significant.
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Figure Legends

Figure 1. Aβ decreases mitochondrial membrane potential in endothelial cells. A, Mitochondrial membrane potential in HUVEC exposed to Aβ (5-50 μM, 30 min), with/without Alda-1 pretreatment (20 μM, 30 min prior Aβ), were measured using the JC-1 probe. Plotted fluorescence units were obtained by subtracting background fluorescence. (**p<0.01 and ***p<0.001 vs. control, n=3). X=Aβ in activity used as control. B, Western blot analysis of cytochrome c release following Aβ treatment (50 μM, 30 min) with/without Alda-1 (20 μM, 30 min prior Aβ). Representative gels of three independent experiments. C, Superoxide mitochondrial generation in HUVEC, detected by MitoSox fluorescence staining. Aβ (50 μM, 30 min), Alda-1 (20 μM, 30 min prior Aβ). Bar length: 100 μm. Quantifications of superoxide generation expressed as % of cells stained with MitoSox ± s.e.m. (n=3). D, Mitochondrial membrane potential in HBMEC exposed to Aβ with/without Alda-1 (20 μM, 30 min prior Aβ), measured using the JC-1 probe and expressed as described in A. **p<0.01 and ***p<0.001 vs. control. Data represents mean ± s.e.m.; n=3-4 emission values. X=Aβ in activity used as control E, Western blot analysis of cytochrome c release in HBMEC treated as above. Gel is representative of three independent experiments. F, Superoxide mitochondrial generation, detected by MitoSox, in HBMEC treated as above. Bar length: 100 μm. Quantifications of superoxide generation expressed as % of cells stained with MitoSox ± s.e.m. (n=3).

Figure 2. Aβ increases 4-HNE adducts formation. A, 4-HNE adducts accumulation in HUVEC treated as in Fig.1A were measured by ELISA. *p<0.05 vs Ctr.; #p<0.05 vs Aβ. Bars are means ± s.e.m. of 3 experiments. B, 4-HNE detection by immunohistochemistry in HUVEC treated as above. C, 4-HNE and TOM20 detection by immunohistochemistry in HUVEC treated as above. Bar length: 10 μm.

Figure 3. Alda-1 prevents Aβ-induced mitochondrial dysfunction. A, JC-1 monomer fluorescence, in HUVEC, induced by Aβ pretreated with Alda-1, in the concentration range 10-40 μM. Data are expressed as in Fig 1 A. Bars are means ± s.e.m., n=3; ***p<0.001. X=Aβ in activity used as control B, Mitochondrial membrane potential were assessed by confocal microscopy, showing the relative abundance of JC-1 aggregates (red) and monomers (green). Control cells (upper panels); Aβ treated cells (middle panels); cells treated with Alda-1-Aβ combined as in Fig. 1A (lower panels). Bar length: 10 μm. E, Phosphatidylinerine
exposure, assessed by immunohistochemistry, in HUVEC treated for 18 hours with Aβ 50 µM with or without Alda-1. Bar length: 100 µm.

**Figure 4.** Aβ inhibits ALDH2 activity in endothelial cells. A, Decline of ALDH2 activity, measured by the formation of NADH in HUVEC exposed to Aβ. Bars are means of NADH production (± s.e.m., n=3-6). B, Western blot analysis of ALDH2 expression in HUVEC treated with Aβ (25 µM) with/without Alda-1 (20 µM). Quantification of gels are presented as a ratio to β actin (n=3). C, Recombinant ALDH2 activity in the absence/presence of Aβ or Alda-1. Data presented are % over control of NADH production ± s.e.m, of 3 independent experiments. D, Expression of ALDH2 and ALDH1 levels in HUVEC by western blot analysis. Known amount of purified recombinant ALDH1 and ALDH2 (1.5-5 ng) were used as a standard curve. Values above blots are quantitated data of three independent experiments. E, Recovery of ALDH2 enzyme activity in HUVEC or F, HMBEC by Alda-1 treatment (20 µM) or MnTbap (100 µM) 30 min before exposure to Aβ (50 µM). **p<0.01 and ***p<0.001 versus control; ###p<0.001 and §p<0.05 versus Aβ.

**Figure 5.** Aβ induces phosphorylation of β-catenin, and impairs endothelial adherens and tight junction organization and barrier function. A, Western blot analysis of phosphorylated β-catenin at ser33/37 and thr41, in HUVEC treated with Aβ (50 µM) in presence/absence of Alda-1 (20 µM). Quantification of gels are presented as a ratio to β actin (n=3) (**p<0.01 vs ctr., ##p<0.01 vs Aβ treatment). B, ZO-1 and VE-cadherin expression pattern in control cells (a and d) or in cells exposed to Aβ (50 µM) (b and e) or in cells pretreated with Alda-1 (20 µM, 30 min) and then exposed to Aβ (c and f). Bar length: 50 µm C, Increased permeability of HUVEC monolayer exposed to Aβ (50 µM) in presence/absence of Alda-1 (20 µM), detected as passage of fluorescence-coniugated FITC-Dextran. Each point is the mean of 3 experiments ± s.e.m., * p < 0.05 versus Aβ.

**Figure 6.** Alda-1 preserves endothelium ability to form pseudocapillary and restores responsiveness to FGF-2. A, Pseudocapillary formation in Matrigel by HUVEC exposed to Aβ (25 µM) with/without Alda-1 (20 µM) or FGF-2 (20 ng/ml), observed by microscopy 18 hr after cell seeding. a, Control (Aβ40-1), b, Aβ, c, combined Alda-1-Aβ treatment, d, FGF-2 treated cells, e, combined FGF-2 and Aβ treatment, f, combined FGF-2, Aβ and Alda-1 treatment. Images represents 3 experiments. Bar length: 20 µm; Insert i and iii: representative
pictures at 4x magnification of pseudocapillary network in control or after FGF-2 treatment.

Insert ii: endothelial cell morphology after 18 hrs of treatment with Aβ. Bar length: 100 μm

B, Quantification of pseudocapillaries. The numbers of circles per well are provided ± s.e.m.; n=3. (***p<0.001 from control; ### p<0.001 from Aβ). C, Formation of pseudocapillaries from HUVEC after 2 days from seeding on cytodex microcarriers embedded in fibrin gel. Representative pictures of capillary formations in control condition (Aβ40-1) (a), after FGF-2 stimulation (b) after FGF-2 + Aβ (c) or FGF-2 + Aβ + Alda-1 (d). Bar length: 50 μm

D, Quantification of pseudocapillaries. The number of grid units required to cover the entire pseudo-capillary surface are reported ± s.e.m.; n=3. (###p<0.001 from control; *** p<0.001 from Aβ).

Figure 7. Alda-1 preserves endothelial cell migration and FGF-2 expression. A, Cell migration measured by Boyden Chamber. Bars are means of 3 experiments (± S.E.M) (**p<0.01 versus control; ### p<0.001 versus Aβ). B, Western blot analysis of FGF-2 production in HUVEC treated with Aβ in presence/absence of Alda-1. Quantification of gels are presented as a ratio to β actin (n=3). *p<0.001.
Figure 1

A (HUVEC)

Mitochondrial potential (fluorescence)

<table>
<thead>
<tr>
<th>Alda-1</th>
<th>Aβ [µM]</th>
<th>5</th>
<th>25</th>
<th>50</th>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tbody>
</table>

Cyt. C/β actin (ADU) | 0.2 ± 0.05 | 0.1 ± 0.06 | 1.5 ± 0.01 | 0.33 ± 0.04
Mitochondrial superoxide production (% of MitoSOX positive cells)

- Ctr.
- Aβ
- Aβ + Alda-1

Mitochondrial potential (fluorescence)

- Alda-1
  - Aβ (μM)
    - 25
    - 50

D HBMEC
Figure 1

E

Mitochondrial superoxide production (% of MitoSox positive cells)

Ctr.  Aβ  Aβ + Alda-1

<table>
<thead>
<tr>
<th></th>
<th>Cyt C</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr.</td>
<td>0.65 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Aβ</td>
<td>2.97 ± 0.02</td>
<td>0.7 ± 0.01</td>
</tr>
</tbody>
</table>

Cyt C/β actin (ADU)
Figure 2

A

B

C

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Figure 3

A

Mitochondrial potential (fluorescence)

Alda-1 (μM)

JC-1 aggregates   JC-1 monomers      merge

Ctr.  Aβ  Aβ + Alda-1

Aβ

JC-1 aggregates   JC-1 monomers      merge

Ctr.  Aβ  Aβ + Alda-1
Figure 3

C

Comparison of cell staining with phosphatidylserine under different conditions:
- Ctr.
- Aβ 50 μM
- Aβ 50 + Alda-1
Figure 4

(A) Recombinant ALDH2 enzymatic activity (nmole/mg protein) were measured in cell lysate of hAD2 fibroblasts with different concentrations of Aβ (μM) in the absence or presence of Alda-1 (20 μM).

(B) Western blot analysis of ALDH2 and β-actin expression. ALDH2/β-actin ratio was calculated for each condition.

(C) Recombinant ALDH2 enzymatic activity (% Control) was measured in cell lysate of hAD2 fibroblasts with different concentrations of Aβ (μM) in the absence or presence of Alda-1 (20 μM).

(D) Western blot analysis of ALDH1 and ALDH2 expression. Purified protein (ng) was measured in cell lysate.
Figure 4
Figure 5

A

β-catenin p S33/37 T41/actin ratio

Ctr. Aβ Alda-1+Aβ

**

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Figure 5

Paracellular flux (fluorescence units)

Time (min)

- Ctr.
- Alda-1
- Aβ
- Aβ + Alda-1
Figure 6

**A**

Control

FGF-2

FGF-2 + Aβ

Aβ + Alda-1

FGF-2 + Aβ + Alda-1

---

**B**

Pseudovascularization formation (Number of circles/well)

- **Aβ 40-1**
- **Aβ**
- **Aβ + Alda-1**

- Significance levels:
  - **@** p < 0.01
  - ******* p < 0.001
  - **###** p < 0.001
Figure 7

A

![Bar chart showing the number of migrated cells per well.](Image)

Migration of Aβ

- Ctr.
- Alda-1

α-actin ratio

B

![Western blot showing FGF-2 and β-actin bands.](Image)

Expression of FGF-2

- Ctr.
- Alda-1
- Aβ
- Aβ + Alda-1

**FGF-2/β-actin ratio**

- **Ctr.**
- **Alda-1**
- **Aβ**
- **Aβ + Alda-1**