Metformin increases APP expression and processing via oxidative stress, mitochondrial dysfunction and NF-κB activation: Use of insulin to attenuate metformin's effect

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ABSTRACT

Clinical and experimental biomedical studies have shown Type 2 diabetes mellitus (T2DM) to be a risk factor for the development of Alzheimer's disease (AD). This study demonstrates the effect of metformin, a therapeutic biguanide administered for T2DM therapy, on β-amyloid precursor protein (APP) metabolism in in vitro, ex vivo and in vivo models. Furthermore, the protective role of insulin against metformin is also demonstrated. In LAN5 neuroblastoma cells, metformin increases APP and presenilin levels, proteins involved in AD. Overexpression of APP and presenilin 1 (Pres 1) increases APP cleavage and intracellular accumulation of β-amyloid peptide (Aβ), which, in turn, promotes aggregation of Aβ. In the experimental conditions utilized the drug causes oxidative stress, mitochondrial damage, decrease of Hexokinase-II levels and cytochrome C release, all of which lead to cell death. Several changes in oxidative stress-related genes following metformin treatment were detected by PCR arrays specific for the oxidative stress pathway. These effects of metformin were found to be antagonized by the addition of insulin, which reduced Aβ levels, oxidative stress, mitochondrial dysfunction and cell death. Similarly, antioxidant molecules, such as ferulic acid and curcumin, are able to revert metformin's effect. Comparable results were obtained using peripheral blood mononuclear cells. Finally, the involvement of NF-κB transcription factor in regulating APP and Pres 1 expression was investigated. Upon metformin treatment, NF-κB is activated and translocates from the cytoplasm to the nucleus, where it induces increased APP and Pres 1 transcription. The use of Bay11-7085 inhibitor suppressed the effect of metformin on APP and Pres 1 expression.

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1. Introduction

The high prevalence of both Alzheimer's disease (AD) and type 2 diabetes mellitus (T2DM) in the elderly population suggests that concomitant pharmacotherapy could be desirable. AD is the leading cause of dementia in the elderly and is characterized by a gradual loss of cognitive function. Aging is the primary risk factor for the development of AD, and for many other pathological conditions (including T2DM), which can obscure AD diagnosis, or interfere with AD pharmacotherapy. Pathohistological hallmarks of AD include widespread neuronal degeneration, extracellular amyloid plaques and intracellular neurofibrillary tangles. Amyloid plaques are produced by pathological deposition of amyloid-β peptide (Aβ) which is a small protein derived from the sequential proteolytic cleavage of amyloid precursor protein (APP) by β-site APP cleaving enzyme 1 (BACE1) and γ-secretase — a multi-subunit protease complex comprised of proteins such as resenilins 1 and 2 (PS1, PS2) [1,2]. Cleavage of APP generates a soluble extracellular fragment, and a cell membrane-bound fragment referred to as C-terminal fragment beta (CTF-b). Cleavage of CTF-b, by γ-secretase, produces Aβ, the accumulation and misfolding of which in neurons lead to the pathological cascade of AD. Fibrillar forms of Aβ found in amyloid plaques were previously considered the major cause of neuronal damage in AD, but Aβ soluble oligomers, known as Aβ derived diffusible ligands (ADDLs), are now considered the more potent neurotoxins [3,4]. The link observed between T2DM and AD [5,6] suggests the existence of common cellular mechanisms. Insulin and insulin-like growth factor-1 (IGF-1) have a direct effect on APP metabolism and Aβ clearance [7]. It has been observed that insulin inhibits Aβ breakdown and exerts this effect via the insulin-degrading enzyme, one of the main proteases involved in Aβ degradation [7]. Clinical findings have indicated that insulin has beneficial effects on cognition in patients with dementia [8], suggesting that insulin signaling may have a neuroprotective effect. A recent study has demonstrated that intranasal

inhalation of insulin improves cognitive function in patients with AD [8, 9]. Investigating interactions between medications for diabetes and AD may clarify the relationship between diabetes, diabetes medications and AD. However, investigations into the potential relationship between antidiabetic drugs and the risk of AD are not well documented, and are limited in that they are based on a handful of conflicting reports from in vitro studies, animal models or limited patient cohort populations [10]. Some findings indicate that metformin (1,2-dimethylbiguanide hydrochloride), the widely prescribed insulin-sensitizing drug, increases the production of Aβ [11], which suggests that its use may promote the development of AD. Furthermore, a neuropathological study has reported that individuals treated both with insulin and oral antidiabetic drugs had a significantly lower amyloid plaque density [12]. A recent population-based case–control study examined the relationship between T2DM and administration of different antidiabetic drugs, and risk of AD development. The results suggested that long-term users of metformin may have a slightly higher risk of AD development relative to the rest of the population [13]. Metformin, one of the most commonly used antihyperglycemic agents, seems to act by triggering AMP activated protein kinase (AMPK), an enzyme that responds to alterations in cellular energy levels [14].

The aim of this study was to determine the molecular mechanisms underlying the effects of metformin on APP metabolism, and of insulin's interference with these processes. The capacity for metformin to modulate APP and presenilin 1 expression via nuclear factor kappa B (NF-κB) activation was explored in order to elucidate these processes.

2. Materials and methods

2.1. Cell cultures and treatments

The neuroblastoma LAN5 cell line was used as cellular model. This cell line exhibits neuronal characteristics, including expression of neurofilament and display of short neuritis. Cells were cultured with RPMI 1640 medium (CELBIO) supplemented with 10% fetal bovine serum (FBS) (GIBCO), 2 mM L-glutamine (SIGMA), and 1% antibiotics (50 mg/ml^{-1} penicillin and 50 mg/ml^{-1} streptomycin) (SIGMA). Cells were maintained in a humidified 5% CO_2 atmosphere at 37 ± 0.1 °C. For dose and time course experiments, LAN5 cells were treated with 12.5, 25, 50, 100 and 200 mM of metformin (SIGMA) in serum free medium for 24 and 48 h, and with 0.1, 1 and 2.5 mM of metformin for 5 or 10 days. In the other experiments, LAN5 cells were treated with 50 mM of metformin. For insulin treatment, cell cultures were treated with metformin (50 mM) combined with insulin at different concentrations (0.5, 1 μM) in serum free medium at 37 °C for 48 h, with 1 mM of metformin and with 10 and 100 nM of insulin for 5 days. H_2O_2 was utilized at 2 and 4 mM for 1 h, with the higher concentration being chosen for experiments in combination with antioxidants. Ferulic acid, a natural antioxidant, was utilized at 25, 50, 75 and 100 μM, in which 50 and 75 μM were chosen as the concentrations for the experiments with metformin and H_2O_2. Curcumin, another natural antioxidant, was dissolved in 100% ethanol, and utilized at 2, 4 and 10 μM, in which 2 and 4 μM were the chosen concentrations for the experiments with metformin and H_2O_2. In experiments with H_2O_2 and metformin, the antioxidant molecules were incubated for 1 h and 24 h respectively. A microscope (Zeiss Axio Scope) with a camera (Axiocam) and 20× and 40× objectives, was utilized to analyze the morphology of the cells. An enzyme-linked immunosorbent sandwich assay (ELISA) (Invitrogen) was performed for quantitative detection of human β-amyloid (1–42) in cell culture supernatants. NF-κB activator (TNF-α) was utilized at 100 U/ml for 24 h. Bay11-7085 (Santa Cruz), an irreversible inhibitor of IκBα phosphorylation was utilized at 0.4 and 0.8 μM for 24 h.

2.2. Determination of cell viability

Cell viability was measured by MTS assay (PROMEGA). 1×10^6/ml LAN5 cells were plated in 96 well plate and after 24 h were untreated (control) or treated with metformin. MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphopheyl)2H-tetrazolium] was used according to the manufacturer's instructions. After cell treatments, 20 μl of the MTS solution was added to each well and incubated with cells for 4 h at 37 °C, 5% CO_2. The absorbance was read at 490 nm with the Microplate reader WallacVictor 2 1420 Multilabel Counter (Perkin Elmer). Results were expressed as the percentage of MTS reduction relative to the control.

2.3. Total protein extraction and Western blotting

1×10^6 LAN5 cells untreated (control) or treated with metformin, alone or with insulin or with antioxidants, H_2O_2 alone or with antioxidants, were harvested using trypsin–EDTA and centrifuged at 500 ×g for 5 min. After washing in PBS and centrifugation at 500 ×g for 5 min the pellets were dissolved in solubilizing buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 1 mM DTT, 0.1% SDS) with protease inhibitor (Amersham) and phosphatase inhibitor cocktails II and III (SIGMA). Mouse brains were homogenized in RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% SDS, and 0.5% deoxycholate) with protease inhibitors (Amersham) and phosphatase inhibitor cocktails II and III (SIGMA). To remove insoluble material, cell lysates were sonicated and centrifuged at 11,500 ×g, for 10 min. Proteins (20 μg) were resolved with NuPAGE 4–12% Bis-Tris gels (Life Technologies) and transferred onto nitrocellulose filters for immunoblotting with anti-β-amyloid (1:500), anti-presenilin 1 (1:500), anti-BACE (1:1000), anti-NF-κB (1:1000), anti-VDAC (1:1000), and anti-Lamin B (1:4000) purchased from Santa Cruz; anti-phosphorylated-AMPK (1:1000), anti-Hexokinase II (1:1000), anti-cytochrome C (1:1000), and anti-phosphorylated NF-κB (1:100) purchased from Cell Signaling; and anti-β-actin (1:1000) purchased from Sigma. Primary antibodies were detected using the ECL chemiluminescence kit (Amersham) according to the manufacturer's instructions and using secondary antibodies conjugated to horseradish peroxidase (1:2000) (Cell Signaling). In some instances, antibodies were stripped from blots with Restore Western Blot Stripping Buffer (Thermo Scientific) for 15 min at room temperature, for antibody reprobing. Band intensities were analyzed with a gel documentation system (BioRad). Expression was normalized with β-actin, Lamin B or VDAC expression.

2.4. ROS generation and mitochondrial membrane potential assays

Following treatment the cells were incubated in the dark with 1 mM dichlorofluorescein diacetate (DCFH-DA) in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_3PO_4, pH 7.4) for 10 min at room temperature, then analyzed with a fluorescence microscope (Axio Scope 2, Zeiss) and a fluorimeter (WallacVictor 2, Perkin Elmer). Mitochondrial membrane potential was measured using a MitoProbe JC-1 Assay Kit (Molecular Probes). After treatment the cells were incubated with 2 mM JC-1 (5,5′,6,6′-tetrachloro-1,1′;,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) fluorescent dye in PBS for 30 min at 37 °C. The mitochondrial membrane potential disrupter CCCP (carbonyl cyanide 3-chlorophenylhydrazone) was used at 50 μM as a control. The shift of JC-1 fluorescence emission from red (590 nm) to green (529 nm) was determined with a fluorimeter (WallacVictor 2, Perkin Elmer).

2.5. Mitochondria isolation

The mitochondria fractions of untreated and treated cells were prepared by using a Mitochondria Isolation kit (Thermo Scientific)

according to the manufacturer's instructions with some buffers provided in the kit. Briefly, 2×10^6 LAN5 cells were pelleted and solubilized in Reagent A. After incubation in ice, Reagent B was added and the sample was centrifuged at 700 ×g for 10 min at 4 °C. The pellet was either discarded or stored (nuclei and cell debris respectively) and the supernatant was centrifuged at 12,000 ×g for 15 min, at 4 °C. The supernatant (cytosol) was either discarded or stored and the pellet was then washed with Reagent C, then centrifuged at 12,000 ×g for 5 min, at 4 °C. The pellet (mitochondrial fraction) was then stored at −80 °C, or used for protein extraction.

2.6. Peripheral blood mononuclear cell isolation

10 ml of venous blood was collected early in the morning from ten healthy donors (age range: 30–40 years old) and peripheral blood mononuclear cell (PBMC) isolation was performed immediately. PBMCs were isolated from heparinized blood of donors by Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB) and cultured at 10^5 cells/well, in a 96 well flat-bottom plate, in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 100 U/ml penicillin/streptomycin at 37 °C. PBMCs were treated with metformin 2.5, 5, 10 and 20 mM and insulin 0.25, 0.5 and 1 μM for 24 h.

2.7. Immunofluorescence

1×10^6/ml LAN5 cells were cultured on Lab-Tek II Chambered Coverglass (Nunc) and treated as described above. After washing in PBS the cells were fixed in 4% paraformaldehyde for 30 min and stored at 4 °C. After incubation with 3% BSA/PBS for 1 h, the cells were then immunostained with anti-phosphorylated-NFκB (1:100; Cell Signaling) antibody at 4 °C overnight. After washing in PBS, the samples were incubated with anti-rabbit TRITC-conjugate secondary antibody (1:300; SIGMA). For nuclear staining, cells were incubated with Hoechst 33258 (5 μg/ml). For detection of mitochondrial activity, one vial of Mito red was dissolved in DMSO according to the manufacturer's instructions (SIGMA). Living cells were incubated with 20 nM Mito red for 5 min. The degree of red staining produced indicated mitochondrial activity. The samples were analyzed by using a DHL fluorescent microscope (Leica) at excitation/emission wavelengths of 350/450 nm, respectively. Depending on the microscopic analysis 20× or 40× objectives were used.

2.8. Preparation of cytoplasmic and nuclear extracts

Nuclear and cytoplasmic extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Thermo Scientific) according to the manufacturers instructions using buffers provided in the kit. Briefly, 2×10^6 LAN5 cells were centrifuged at 500 ×g for 5 min at 4 °C. The pellet was suspended in ice-cold CERI buffer. After 10 min, CERII buffer was added and the sample was centrifuged at 16,000 ×g for 5 min at 4 °C. The supernatant (cytoplasmic extract) was stored at-80 °C and the pellet was suspended in NER buffer. After centrifugation at 16,000 ×g for 10 min at 4 °C, the supernatant (nuclear extract) was stored at-80 °C.

2.9. Quantitative Real-Time qPCR

Total RNA was extracted using the RNEasy Mini Kit (Qiagen). Two nanograms of RNA was used to synthesize the first strand cDNA using the RT First-Strand kit (Qiagen). Synthesized cDNAs were amplified using the RT^2 SYBR Green/ROX qPCR Mastermix (Qiagen) and StepOne Real-Time instrument (Applied Biosystem). Gene expression validation was performed using RT^2 qPCR Primer Assay for human APP, presenilin 1, NOX2, NOX5, COX1, COX2, GPX7, GSS, GSTP1, SOD3, and β-actin (SABiosciences). Gene expression was normalized to β-actin.

2.10. RT^2 Profiler PCR array

Human oxidative stress (PAHS-065Z) focused pathway arrays RT^2 Profiler PCR Array Human Oxidative Stress (SABiosciences) in 96-well plate format, were used to assay gene expression changes. Samples were prepared from pooled RNA extracted from metformin untreated and treated LAN5 cells. Samples were added to the reaction plates according to the manufacturer's instructions and a StepOne Real-Time instrument (Applied Biosystem) was used to perform the array. Analysis was performed using the spreadsheet provided by SABiosciences.

2.11. Mice

The animal studies were approved by Ministero della Sanità (Rome, Italy) and in compliance with the guidelines of the European Communities Council Directive of 24 November 1986. Male C57B6/J (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone Udine, Italy) at 4 weeks of age, were housed under standard light (12 h light:12 h darkness cycle) and temperature (22–24 °C) conditions. After acclimatization (1 week), the animals were divided into two groups (control and metformin treated). Mice were provided food and received metformin in drinking water (2 mg/ml) for 7 days. After this period of treatment animals were sacrificed by cervical dislocation.

2.12. Statistical analysis

All experiments were repeated at least three times and each experiment was performed in triplicate. The results are presented as mean ± SD. A one-way ANOVA was performed, followed by Dunnett's post hoc test for analysis of significance. Results with a P-value < 0.05 were considered statistically significant, *P < 0.05 and **P < 0.02.

3. Results

3.1. Metformin increases Aβ metabolism and APP and presenilin 1 expression

LAN5 neuroblastoma cells were used as an initial model to test the effect of metformin on APP metabolism. Cells treated with metformin showed differing degrees of degeneration and morphological changes resulting in reduction of the cell body, neurites and cell number (Fig. S1A). Decreased viability in a dose and time dependent manner was confirmed by MTS assay (Fig. S1B). Furthermore, incubation with Hoechst 33258 revealed the presence of DNA nicks, a hallmark of apoptosis, in the metformin treated sample (Fig. S1C).

To examine the effect of metformin on APP and various metabolite expression levels, and the presence of Aβ aggregates, a Western blot of proteins extracted from LAN5 cells treated with differing metformin concentrations and exposure times was incubated with an anti-Aβ antibody raised against the 42 amino-acids of Aβ. Higher metformin concentrations correlated with increased expression of APP and, consequently, the formation of Aβ fragments and aggregates (Fig. 1A). An intriguing observation was that as metformin concentration and exposure time increased, growth of larger and larger Aβ aggregates was observed. In order, to analyze whether metformin also has an effect on secretases involved in APP processing, the same Western blot was incubated with anti-presenilin. Its expression levels increased in a dose and time dependent manner (Fig. 1A). In accordance with the protein levels observed, expression of APP and presenilin mRNAs, analyzed by quantitative real-time PCR (qRT-PCR), was significantly increased in metformin treated LAN5 cells relative to controls (Fig. 1B). Further, the effects of metformin concentration have been explored for prolonged exposure ranged from 0.1 to 2.5 mM. After 10 days of incubation the lower dose utilized (0.1 mM) showed a rate of mortality comparable to the rate observed at 100 mM for 24 h (Fig. S1B, E). Regarding the effect on the percentage of cell viability (Fig. S1B, E), APP and presenilin
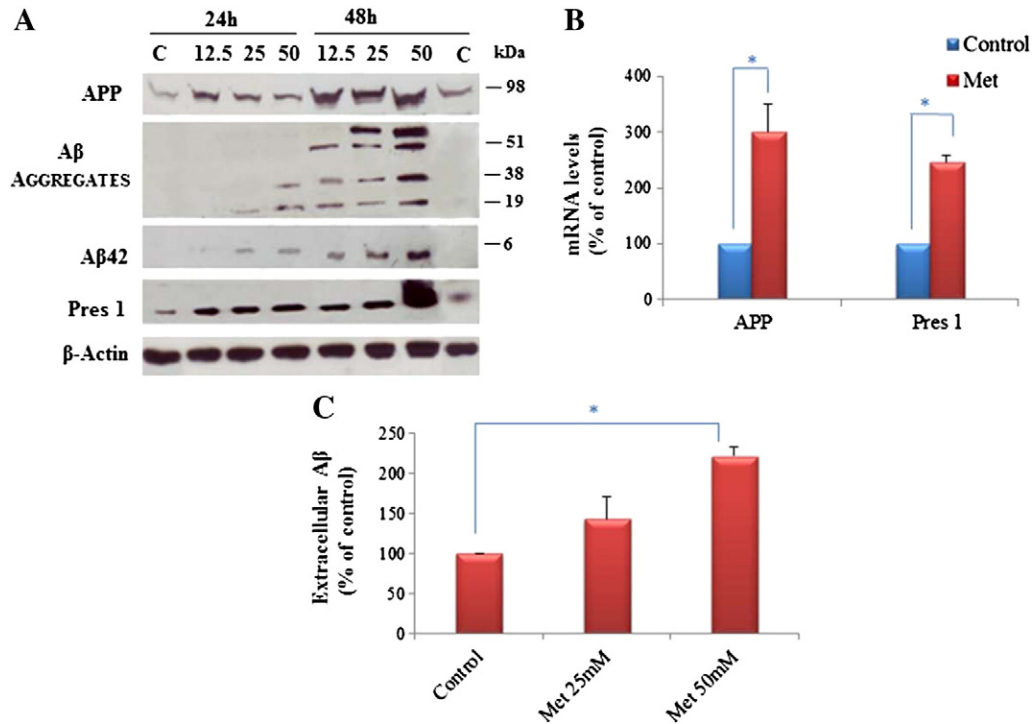
Fig. 1. A) Analysis of APP and its metabolites, including Aβ42 and Aβ aggregates and presenilin 1 (Pres 1) upon metformin treatment. Western blot of proteins extracted from LAN5 control (C), or treated with metformin at different concentrations (12.5, 25, 50 mM) for 24 and 48 h. Uniformity of gel loading was confirmed with β-actin utilized as standard. The molecular weigh markers (in kDa) used in PAGE are indicated on the right. B) Effect of metformin on APP and presenilin 1 (Pres 1) mRNA levels. The APP and presenilin transcript levels were determined by quantitative real-time PCR. C) Dose dependent metformin's effect at 24 h on extracellular Aβ measured by ELISA. *P < 0.05, **P < 0.02, versus indicated groups.

expression, and APP processing, comparable responses were observed between metformin treatment with 1 mM for 5 days and with 50 mM for 24 h (Fig. S2A, B; Fig. 1). To obtain significant responses in a reasonable observation time most of the following experiments were performed using a concentration of 50 mM for 24 h. Furthermore, to determine whether the Aβ generated was also secreted into the extracellular environment, an ELISA assay was performed in supernatant of LAN5 cells treated with metformin for 24 h. An increase in production of secreted Aβ in a dose dependent manner, occurring the maximum effect (about 2.5-fold) at 50 mM, was observed (Fig. 1C).

3.2. Insulin protects LAN5 cells against metformin toxicity

Insulin's cascade protects against Aβ-toxicity [15,16] and, in this context, we investigated whether insulin can mediate the effects of metformin on APP metabolism. LAN5 cells with metformin and differing concentrations of insulin, led to a recovery of viability (Fig. 2A) and of cell morphology (Fig. 2B). Furthermore, after the addition of insulin, a significant inhibition of metformin-stimulated Aβ production and presenilin levels, in a dose-dependent manner, was observed (Fig. 2C, D). In accordance with previous results, the increased expression of APP and presenilin mRNA in metformin treated LAN5 cells was reduced by the presence of insulin, in a dose-dependent manner, as demonstrated by qRT-PCR analysis (Fig. 2E, F). Furthermore, we determined that lower concentrations of insulin were required for it to exert its protective role when minor metformin doses were administered for 5 days (Fig. S3).

3.3. Insulin reduces oxidative stress and mitochondrial dysfunction generated by metformin

Oxidative stress is one of the main causes of cell death. Insulin is able to inhibit this process [17,18]. We investigated whether metformin's toxicity is induced through oxidative stress that can, in turn, be antagonized by insulin. Fluorimetric analysis demonstrated that the presence

of intracellular ROS, increased proportionally to metformin concentration (Fig. 3A). The effect of metformin was reversed by the addition of insulin, indicated by a dose dependent manner decrease in DCF fluorescence intensity (Fig. 3B). A physiological fluorescent signal was detected in control cells. Mitochondrial membrane potential (ΔΨ$_m$) is critical for maintaining the physiological function of the respiratory chain to generate ATP. To detect the status of mitochondrial membrane potential (ΔΨ$_m$) we performed a JC-1 assay, in which membrane depolarization is measured and presented as differing red and green fluorescence intensities. In samples treated with metformin and CCCP, green fluorescence was higher than red fluorescence, indicating a significant decrease in ΔΨ$_m$ (Fig. 3C). In contrast to this, a strong decrease in green fluorescence was detected in samples in which insulin had been added, indicating that the physiological mitochondrial membrane potential was re-established (Fig. 3D). To further confirm the inhibition of metformin mitochondrial damage by insulin the cells were stained with Mito Red dye to determine the level of respiratory activity. Further to this, the fluorescent dye Hoechst 33258 was used to investigate whether condensed chromatin, characteristic of apoptotic cells, was present. In cells treated with metformin no mitochondrial activity was observed. However, cells treated with both metformin and insulin showed strong red staining, which, together the absences of DNA nick, suggests that insulin attenuated the effect of the drug (Fig. 3E).

The mitochondrial collapse of the ΔΨ$_m$ is a typical phenomenon following opening of the permeability transition pores (PTPs) of the mitochondrion. This causes the release of cytochrome C, a key event in the mitochondrial pathway of apoptosis. To test whether metformin can affect HK-II, a protein of the PTP complex, and, consequently, cytochrome C, fractionation of LAN5 cells treated with metformin without or with insulin was performed to produce cytosolic and mitochondrial fractions. A decrease in mitochondrial HK-II and cytochrome C levels was observed in samples treated with metformin (Fig. 3F–H). In the presence of insulin both HK-II and cytochrome C levels were comparable to those of the control (Fig. 3F–H).
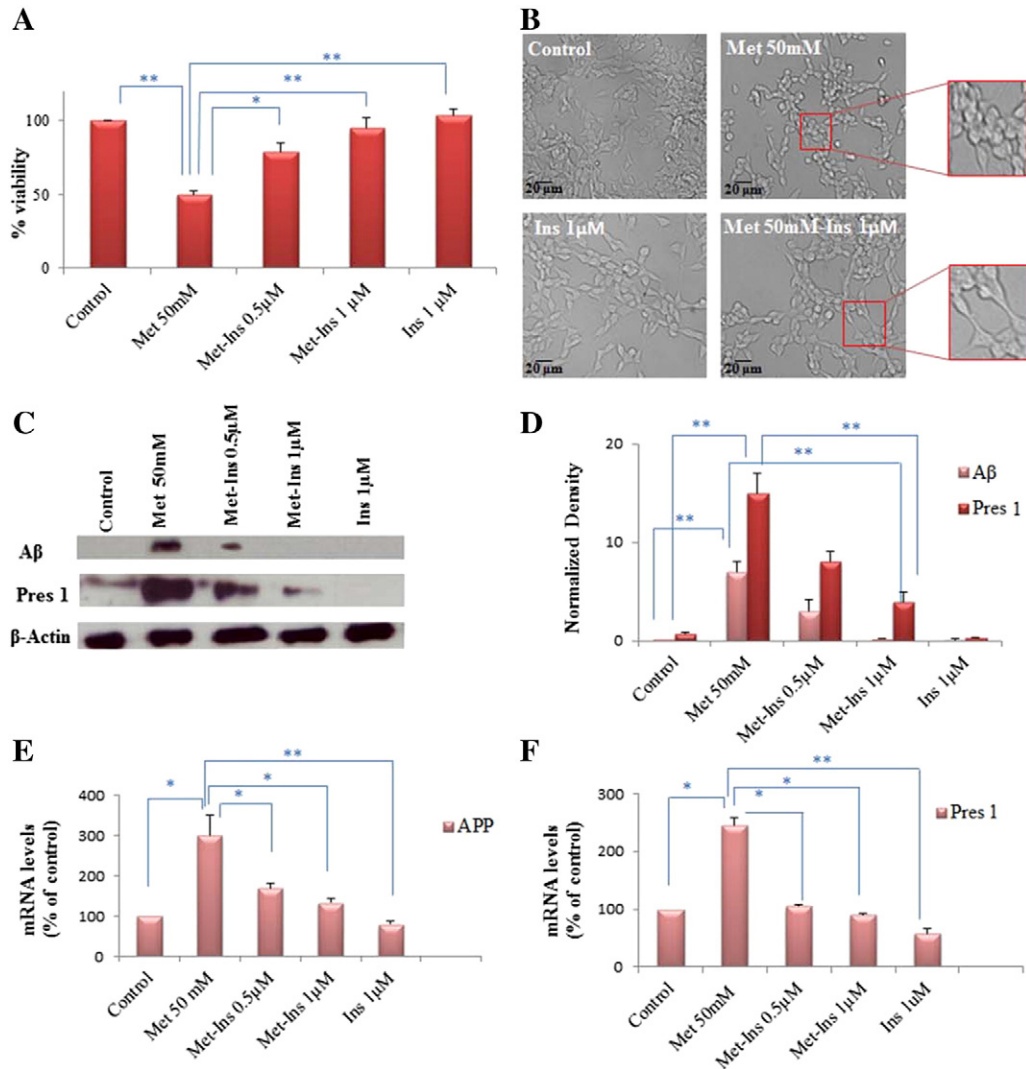
Fig. 2. Insulin protects neuroblastoma cells against metformin toxicity and reduces Aβ generation. A) LAN5 neuroblastoma cells treated with metformin (Met) at 50 mM alone or with insulin (Met-Ins) at 0.5 and 1 μM, or insulin (Ins) alone for 48 h and submitted to MTS assay. The viability is expressed as the percentage as MTS reduction with respect to the control cells. B) Representative morphological images of LAN5 untreated cells (control), or treated with metformin (Met) at 50 mM alone or combined with insulin (Met-Ins) at 1 μM, or insulin (Ins) alone, for 48 h. Higher-magnification images of degenerated and recovered neurons are represented in the squares. Bar, 20 μm. C) Western blot of proteins extracted from LAN5 untreated or treated with metformin (Met) at 50 mM alone or with insulin at 0.5, 1 μM or with insulin alone and incubated with anti-APP and anti-presenilin 1 (Pres 1); uniformity of gel loading was confirmed with β-actin utilized as standard. D) Quantification of immunoreactivity was performed using densitometric analysis. Insulin inhibits APP and presenilin transcripts. E, F) Effect of insulin on reducing metformin's outcome on APP and presenilin 1 (Pres 1) mRNA levels. The APP and presenilin 1 transcript levels were determined by quantitative real-time PCR. *P < 0.05, **P < 0.02 versus indicated groups.

3.4. Altered expression of oxidative stress-related genes in metformin treated cells

To confirm that metformin acts as a pro-oxidant molecule, we analyzed the expression of oxidant-related genes by employing gene expression profiling technology. We compared the expression levels of 84 genes that regulate oxidative stress, including antioxidant genes and those involved in ROS and superoxide metabolism (Figs. S4 and 4A). Twenty-three of the 84 genes had expression levels that were increased or decreased by more than 2-fold in metformin treated samples relative to the control (Fig. 4B). In addition, we validated the expression of NOX2, NOX5, COX1, and COX2, genes associated with ROS production and of GPX7, GSS, GSTP1, and SOD3, genes associated with antioxidant function by quantitative real-time PCR (Fig. 4C, D). As shown, the observed changes were always of the same order of magnitude as those obtained by PCR Arrays and were in agreement with the oxidative stress status of the cells. Therefore, oxidative stress induced by metformin up-regulates ROS metabolism genes and down-regulates antioxidant genes.

3.5. Metformin produces oxidative stress and increases APP expression in PBMC

Human lymphocytes have been utilized to study inflammatory response, oxidative stress, apoptosis and specific signaling in the presence of Aβ [19,20]. To validate our observation about the injurious effect of metformin on neuronal cultures, we treated peripheral blood mononuclear cells (PBMC) from healthy young people, with a range of concentrations (5, 10, 20 mM) of this drug. In the presence of metformin cell viability was lowered by 35–45% relative to the control, with a weak dose dependency (Fig. 5A). Consistent with our previous results, the toxicity induced by metformin was antagonized by insulin addition (Fig. 5B). Furthermore, fluorimetric analysis and microscopic observation revealed increased levels of ROS, indicating that the same molecular drug response was occurred in the ex vivo system (Fig. 5C, D). As expected, reduction in ROS was observed in the presence of insulin (0.25, 0.5, 1 μM) (Fig. 5E). The effect on APP and presenilin expression in PBMC was analyzed and found to be increased by metformin. This
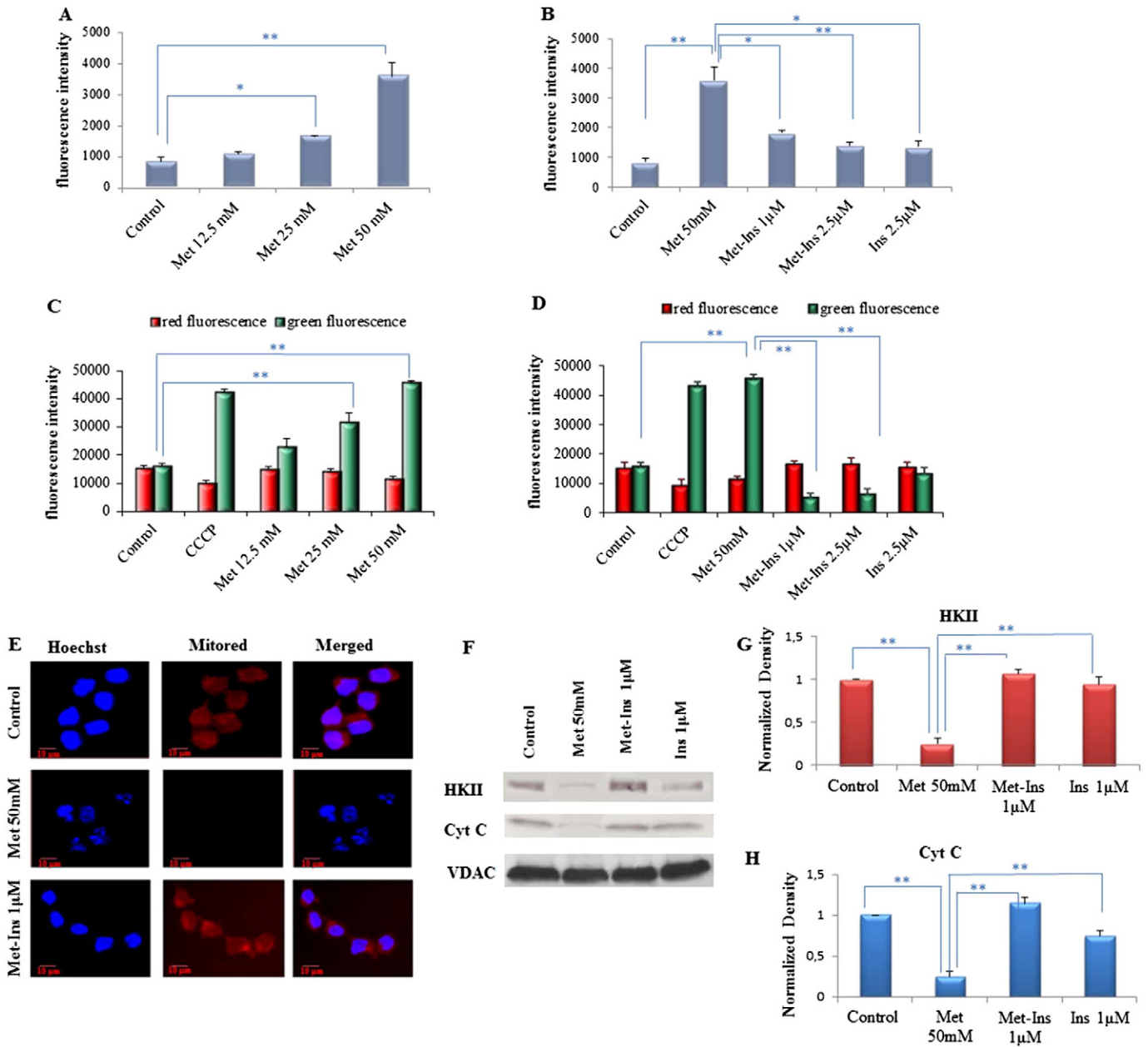
Fig. 3. Metformin induces ROS generation and mitochondrial dysfunction in neuroblastoma cells in dose-dependent manner. A) LAN5 cells were untreated (control) or treated with metformin (Met) (12.5, 25, 50 mM) for 24 h. B) LAN5 cells were treated with metformin (Met) at 50 mM alone or with insulin (Met-Ins) at 1 and 2.5 μM or with insulin alone (Ins 2.5 μM) for 24 h. After these treatments the cells were submitted to DCFHDA assay and fluorescence was measured. C) Untreated LAN5 cells (control) or treated with metformin (Met) (12.5, 25, 50 mM) or with CCCP, as positive control. (D) Untreated LAN5 cells (control), treated with metformin (Met) at 50 mM, with metformin and insulin (Met-Ins) at 1 and 2.5 μM, with insulin alone (Ins 2.5 μM) or with CCCP as positive control. After these treatments the cells were submitted to JC-1 assay. The histograms in C and D represent the obtained difference between red and green fluorescence intensity. E) Insulin recovers mitochondrial respiratory activity, reduced by metformin. LAN5 cells were untreated (control), or treated with metformin (Met) at 50 mM, or with metformin and insulin (Met-Ins) at 1 μM, and incubated with Mito Red and Hoechst 33258. The images of each sample were merged. Red staining indicates mitochondrial activity while intense blue staining indicates nuclear fragmentation. Bar, 10 μm. Metformin modulates Hexokinase II and cytochrome C levels. F) Western blot of proteins extracted from mitochondria of untreated LAN5 cells (control) or treated with metformin (Met) (50 mM), with metformin and insulin (Met-Ins) (1 μM) or with insulin alone (Ins) and incubated with, anti-Hexokinase II (HKII) and anti-cytochrome C (Cyt C). Uniformity of gel loading was confirmed with VDAC utilized as standard. G, H) Quantification of immunoreactivity was performed using densitometric analysis. *P < 0.05, **P < 0.02 versus indicated groups.

increase was found to be attenuated by the presence of insulin (Fig. 5F–H), indicating a strong correlation in the drug response.

3.6. Antioxidants revert APP levels induced by H_2O_2 and metformin

To verify our observation that metformin affects APP metabolism via oxidative stress activation, we tested whether other oxidant molecules such as H_2O_2 have a comparable effect. LAN5 cells were treated with two concentrations of H_2O_2 and the consequent effect on APP protein levels was evaluated by immunoblotting. As shown in Fig. 6A and B,

APP levels increased in the presence of H_2O_2 in a dose dependent manner. Following this, we tested whether antioxidant molecules could antagonize the oxidant effect observed. For this aim we chose two different antioxidants ferulic acid (FA) and curcumin. FA is a molecule naturally present in plant cell walls with high antioxidant and anti-inflammatory properties [21]. Curcumin, a natural polyphenol extracted from the rhizome of *Curcuma longa*, has a wide range of pharmacological activities such as anticancer, antioxidant and anti-inflammatory activities [22]. Optimal FA and curcumin concentration was determined using viability experiments on LAN5 cells (Fig. S5). LAN5 cells were
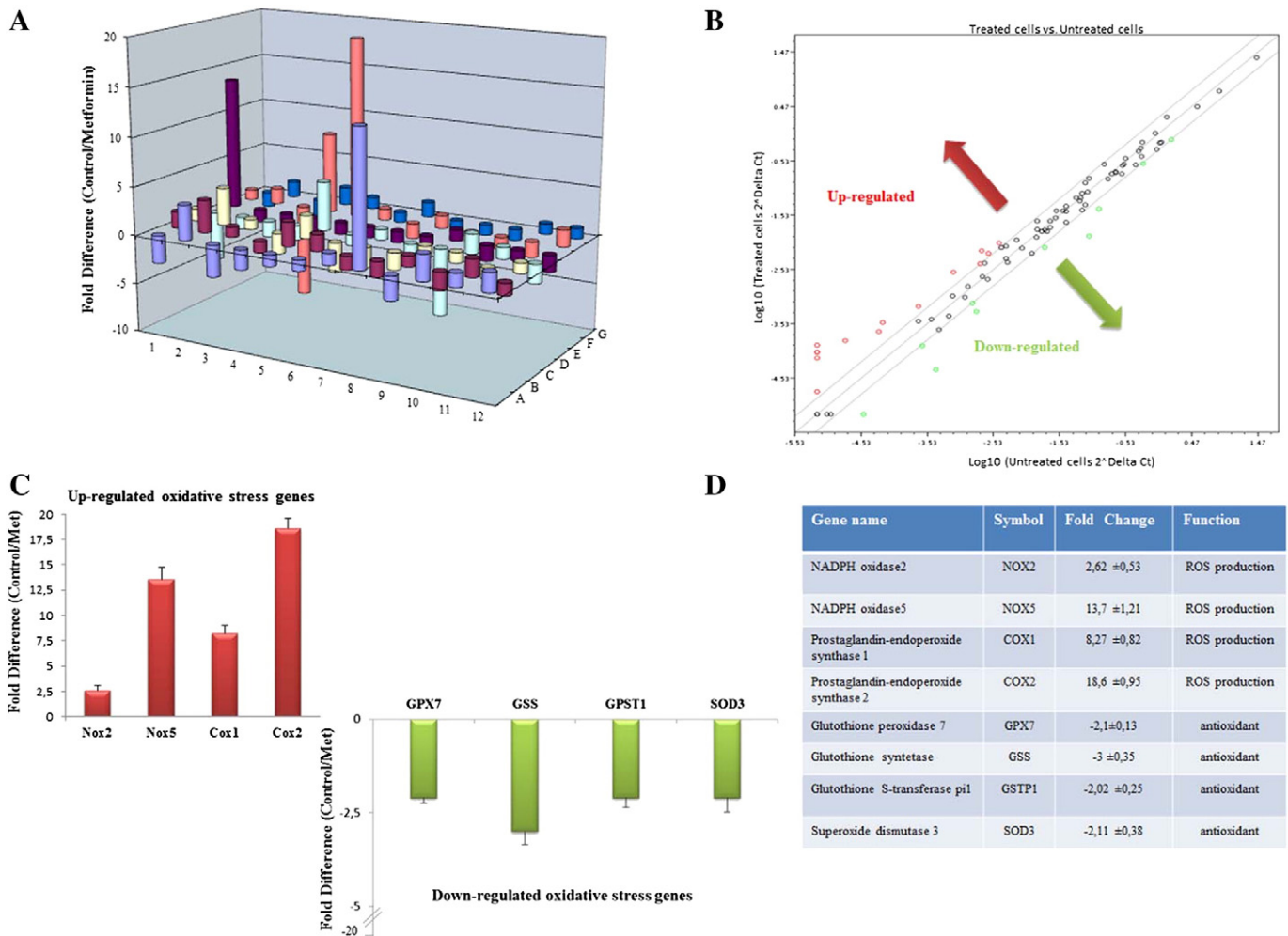
Fig. 4. Oxidative stress PCR Array analysis. Untreated and treated with metformin LAN5 cells were used to prepare the mRNA for the PCR Array analysis. A) Three-dimensional chart taken from SABiosciences analysis software showing the fold difference in expression levels for 84 oxidative stress-related genes (metformin vs control). Position of the genes is signified in Fig. S2. B) Scatter Plot of relative expression levels for each gene in the two samples (metformin vs control). The figure depicts a log transformation plot of the relative expression level of each gene ($2^{-\Delta Ct}$) between untreated cells (x-axis) and treated cells (y-axis). The gray lines indicate a two-fold change in gene expression threshold. Red rings indicate up-regulated genes, green rings indicate down-regulated genes. C) Histogram of some genes with a greater than 2-fold expression change chosen to validate PCR Array. D) Table of quantitative real time PCR analysis ($\pm$SD) of the chosen genes.

incubated with H_2O_2, or metformin combined with FA or curcumin, at two different concentrations. The APP levels were then examined. As expected the antioxidants were able to reduce the effect of both H_2O_2 and metformin on APP production (Fig. 6C–F).

3.7. Metformin activates NF-κB and leads to its translocation from the cytoplasm to the nucleus

NF-κB is a key transcription factor implicated in the regulation of many genes in both cytoprotective and apoptotic pathways. In the conventional pathway of NF-κB activation, phosphorylation of NF-κB's inhibitor (IκBα) releases it from NF-κB, allowing its degradation and consequently, NF-κB activation and translocation to the nucleus. Cytokines such as tumor necrosis factor (TNF) and other stressors activate NF-κB, which translocates from the cytoplasm to the nucleus where it initiates specific gene expression. Recent studies have indicated that APP and presenilin expression can be regulated by NF-κB [23]. As metformin causes ROS production, we investigated whether it also causes subsequent activation of NF-κB. Levels of activated NF-κB were increased in LAN5 cells treated with metformin, or with TNF-α (Fig. 7A, B). These results were confirmed by immunofluorescence analysis. Active NF-κB was undetectable in the control cells, whereas strong expression of NF-κB was detected in the nuclei of metformin and TNF-α LAN5

treated cells (Fig. 7C). As a consequence of the previous experiments, we evaluated the shuttling of NF-κB between the cytoplasm and the nucleus following treatment with metformin and TNF-α as a positive control. For this aim, LAN5 cells, after the specified treatments, were separated into cytosolic and nuclear fractions. Protein extracts were immunoblotted with antibodies raised against total NF-κB and laminin B, as a nuclear control. As expected, NF-κB was mainly present in the cytoplasm fraction of control cells, whereas an increased level in the nuclear fraction was detected in metformin and TNF-α treated samples (Fig. 7D, E). Consistent with the data reported that demonstrates that metformin activates APP and presenilin gene expression, we explored the involvement of NF-κB as their transcription factor. Bay11-7085, an irreversible inhibitor of IκBα phosphorylation, was added at different concentrations of metformin treated samples. qRT-PCR analysis showed an inhibition both of APP, and of presenilin transcription in a dose dependent manner. The higher dose, in particular, caused a reduction of 75% of APP and presenilin expression (Fig. 7F, G). This result denotes the existence of an NF-κB dependent mechanism for metformin's effect on Aβ generation.

Finally, we investigated whether insulin and the antioxidants used in our earlier experiments could inhibit the effect of metformin on NF-κB. Protein extracted from LAN5 cells treated with metformin and either insulin or FA and curcumin were immunoblotted with an antibody
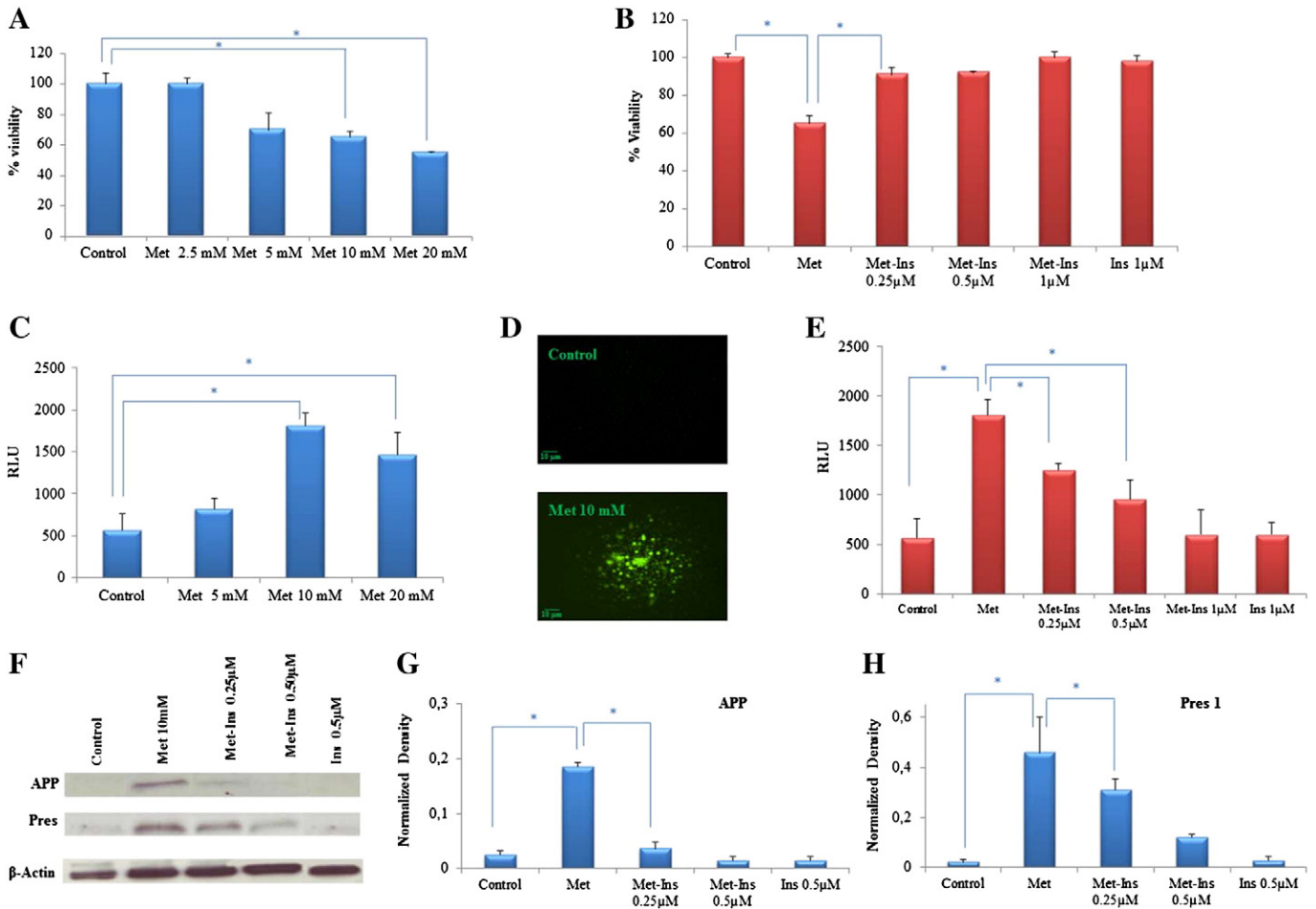
Fig. 5. Metformin induces toxicity in PBMCs, via ROS generation, recovered by insulin. A) PBMCs were untreated (control) or treated with metformin (Met) at different concentrations (2.5, 5, 10, 20 mM) for 24 h and submitted to MTS assay. B) PBMCs were untreated (control) or treated with metformin (Met 10 mM) and with different insulin concentrations (Met-Ins 0.25, 0.5, 1 μM) or with insulin alone (Ins 1 μM) and submitted to MTS assay. Metformin produces ROS generation in PBMCs. C) PBMCs were untreated (control) or treated with metformin (Met) at different concentrations (5, 10, 20 mM) and submitted to DCFHDA assay. D) Representative fluorescent images of untreated LAN5 cells (control) or treated with metformin (Met). Bar, 10 μm. E) PBMCs were untreated or treated with metformin (Met 10 mM) alone or with different insulin concentrations (Met-Ins 0.25, 0.5, 1 μM) or with insulin alone (Ins 1 μM) and submitted to DCFHDA assay. Metformin induces APP and presenilin expressions that are inhibited by insulin. F) Western blot of protein extracted from PBMCs untreated (control) or treated with metformin alone (Met 10 mM) or with insulin at two concentrations (Met-Ins 0.25, 0.5 μM) or with insulin alone (Ins 0.5 μM) and incubated with anti-APP and anti-presenilin 1 (Pres 1). Uniformity of gel loading was confirmed with β-actin as standard. G, H) Quantification of immunoreactivity, was performed using densitometric analysis. *P < 0.05, versus indicated groups.

raised against phosphorylated NF-κB. All the administered substances were able to inhibit activation of NF-κB induced by metformin (Fig. 8A, B). To confirm our previous results, we used immunofluorescence analysis to investigate whether active NF-κB was present into the nucleus (Fig. 8C). Intense immunostaining was detected in the nuclei of metformin treated LAN5 cells, whereas active NF-κB levels were undetectable in cells treated with metformin and either insulin or ferulic acid and curcumin, providing evidence that NF-κB activation is induced by oxidative stress caused by metformin.

3.8. Metformin can reach the mouse brain and increase APP levels

To validate our observations obtained from neuronal cultures and PBMC, and to verify whether the drug is able to reach the brain, we systematically treated C57B6/J mice with metformin (2 mg/ml in drinking water) for 7 days, a dose comparable to 300 mg/kg/day [11]. To confirm deliver of metformin to the brain we tested its effect on AMP-activated protein kinase (AMPK). AMPK is a major cellular regulator of lipid and glucose metabolism, and considered one of the main molecular targets of metformin [14]. Levels of AMPK's phosphorylated form were correlated with increased levels of APP and BACE indicating modification of APP metabolism by metformin in the mouse brain (Fig. 8A–D). Finally,

expression of APP and presenilin mRNAs, quantified using qRT-PCR, was significantly increased in metformin treated mice relative to the control (Fig. 8E), suggesting that the drug exerts a similar effect in vitro, ex vivo and in vivo.

4. Discussion

The molecular mechanism that may link metformin use with increased risk of AD development, the protective role that insulin and/or antioxidants may have when used in combination with metformin, and the involvement of the transcription factor NF-κB in APP and presenilin modulation in these processes was investigated.

Our findings indicate that metformin upregulates APP and presenilin-1 gene expression and consequently increases intra- and extra-cellular Aβ via a mechanism involving mitochondrial dysfunction, free radical generation and cell death. In addition, since it is well known that oxidative stress induces Aβ peptide production that, in turn, triggers oxidative stress, our studies suggests that metformin contributes to the vicious cycle by which Aβ self-feeds its own production [24,25]. Moreover, although the precise mechanism is undetermined we cannot exclude that the high concentration of Aβ peptide induces its conformational change and consequent aggregation. Furthermore, the increase of APP
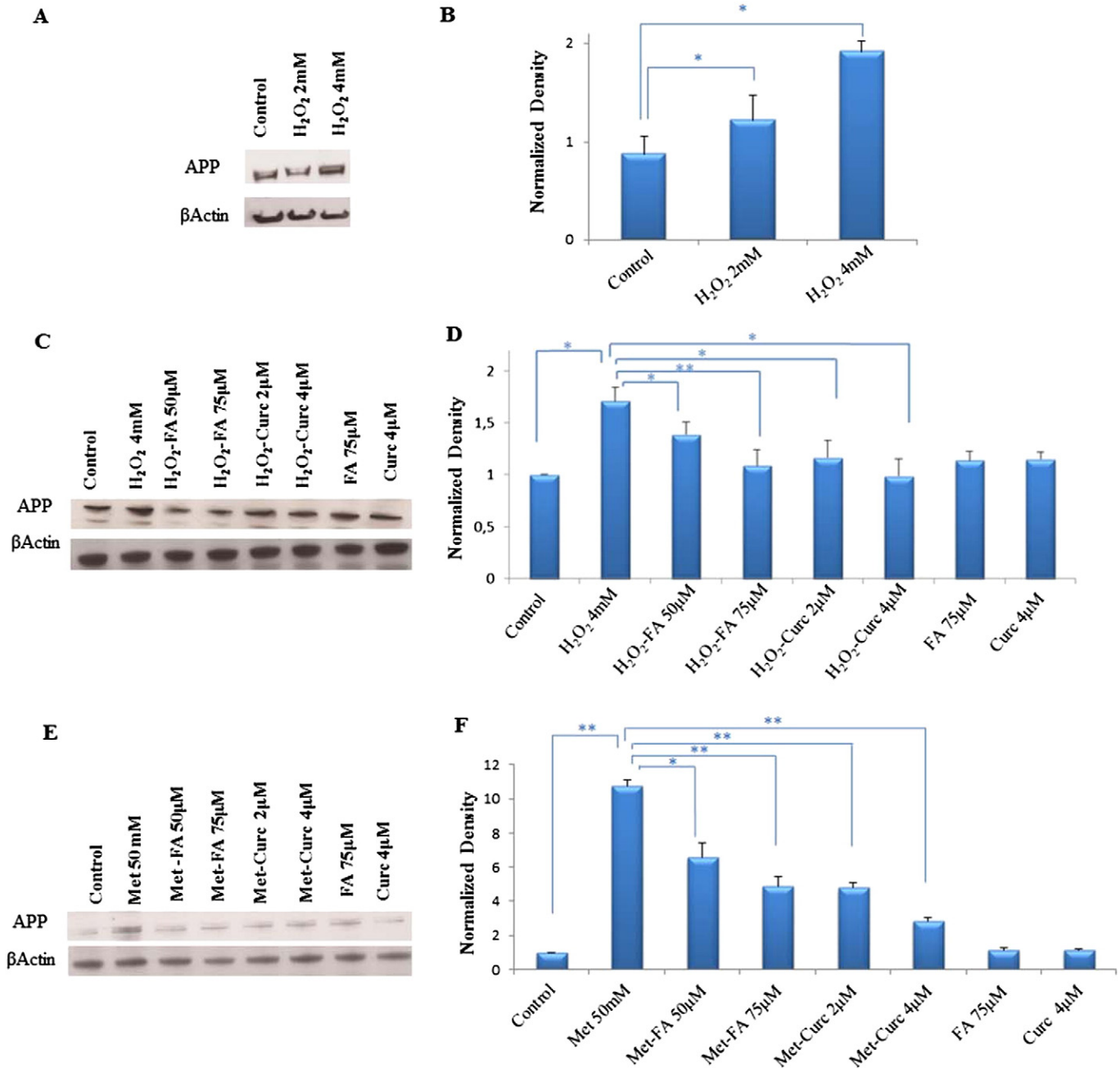
Fig. 6. Antioxidants inhibit production of APP. A) Western blot of proteins extracted from LAN5 cells treated with H_2O_2 at different concentration and incubated with anti-APP. C) Western blot of proteins extracted from LAN5 cells treated with H_2O_2 (4 mM) and FA (50, 75 μM) and curcumin (2, 4 μM) at different concentrations and incubated with anti-APP. E) Western blot of proteins extracted from LAN5 cells treated with Metformin (50 mM) and FA (50, 75 μM) and curcumin (2, 4 μM) at different concentrations and incubated with anti-APP. Uniformity of gels loading was confirmed with β-actin utilized as standard. B, D, F) Quantification of immunoreactivity, was performed using densitometric analysis; *P < 0.05, **P < 0.02 versus indicated groups.

production and processing is counteracted by the presence of insulin. Some studies suggest that insulin influences APP-Aβ metabolism by accelerating trafficking from the trans-Golgi network to the plasma membrane [26], and that insulin administration suppresses the expression of APP, presenilins 1 and 2, and Gsk-3β in PBMC of obese type 2 diabetic patients [27].

Metformin acts as an oxidant molecule, having the same consequence of H_2O_2 on APP metabolism which is antagonized by antioxidant molecules such as FA and curcumin. This result is in agreement with the experimental observation that antioxidants are able to reduce Aβ — induced oxidative stress [28–31]. Metformin induces mitochondrial membrane depolarization (decrease of $\Delta\Psi_m$) in contrast, the presence of insulin leads to repolarization of the mitochondrial membrane

(recovery of $\Delta\Psi_m$). Metformin has been shown to inhibit mitochondrial complex I activity leading to impairment of mitochondrial function [32, 33] and also to prevent PTP opening in both permeabilized and intact cells [34,35]. However, other authors observed that metformin exacerbates PTP opening in isolated rat mitochondria, predisposing to cell death [36,37]. As HK-II is a constituent of mitochondrial PTP, its reduction following metformin administration, suggests that the drug may induce cytochrome C release. Thus, metformin induces mitochondrial dysfunction that together to oxidative stress is one of the primary events of AD onset. However, these effects of metformin were reverted with the addition of insulin. Insulin can exert a potent redox-regulating role through the attenuation of ROS generation and the elimination of ROS and reactive nitrogen species (RNS) by exercising a scavenger
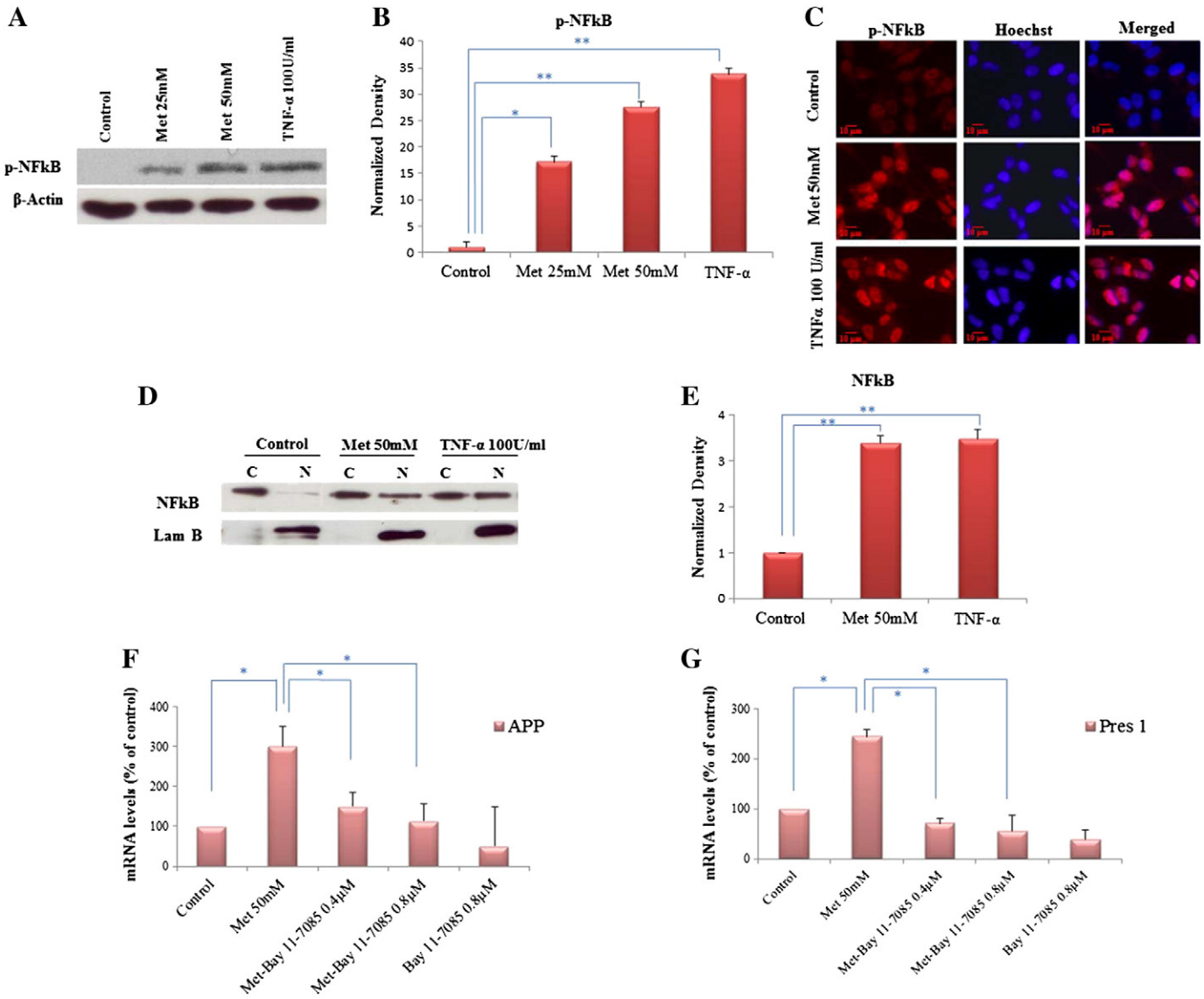
Fig. 7. Nuclear-cytoplasmic shuttling of NF-κB. A) Western blot of proteins extracted from LAN5 cells untreated (control) or metformin-treated (Met) at 25 and 50 mM or treated with TNF-α and incubated with anti-phospho-NF-κB (p-NFκB). Uniformity of gel loading was confirmed with β-actin as standard. B) Quantification of immunoreactivity was performed using densitometric analysis. C) Immunofluorescence of LAN5 untreated (control) or metformin (Met) and TNF-α treated cells, incubated with anti-phospho-NF-κB (p-NFκB) (red staining) and Hoechst 33258 (blue staining) and examined by fluorescent microscopy. Merged images of anti-phospho-NF-κB and Hoechst 33258 staining are shown. Bar, 10 μm. D) Western blot of proteins extracted from LAN5 cells untreated (control) or metformin (Met) and TNF-α treated and subjected to subcellular fractionation in cytoplasmic (C) and nuclear (N) fractions and incubated with anti-NF-κB (NFκB). The relative purity of the nuclear and cytoplasmic fractions was confirmed by probing with the nuclear marker laminin B. E) Quantification of immunoreactivity was performed using densitometric analysis. NF-κB inhibitor reduces APP and presenilin 1 transcription induced by metformin. LAN5 cells were untreated (control) or treated with metformin (Met) alone or with metformin with two Bay11-7085 concentrations or with Bay11-7085 alone and the APP (F) and presenilin 1 (Pres 1) (G) mRNA were quantized. Transcript levels were determined by quantitative real-time PCR. *P < 0.05 **P < 0.02 versus indicated groups.

function [38]. In support of this finding, insulin signaling, via Akt activation, has been found to be involved in the antagonism of oxidative stress induced by Aβ through inhibition of FoxO3a, a pro-apoptotic transcription factor stimulated by ROS generation [16]. Similarly, insulin attenuates mitochondrial dysfunction in neuronal cells and in the kidney and heart isolated from diabetic rats [16,39,40]. Further evidence that metformin induces cell degeneration via oxidative stress is in the modulation of genes related to oxidative stress in AD. The stressed status of the cell was evident in the modulation of these genes, since genes involved in ROS production were up-regulated genes involved in antioxidant function were down-regulated. NADPH oxidase (NOX), a mitochondrial membrane enzyme, contributes to generation of superoxide and other downstream ROS [41]. In particular, NOX2 was found localized in the cerebral cortex and hippocampus of the AD brain [42]. Cyclooxygenase (COX) is an enzyme that converts arachidonic acid to prostaglandins with two known isoforms. COX-1 plays a constitutive

"housekeeping" role. COX-2, is an inducible isoform, the expression of which has been correlated with amyloid plaque density [43]. Physiological antioxidant molecules counteract oxidative stress. Glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase are the main enzymes involved in cellular protection against damage due to oxygen-derived free radicals [44]. GPxs (1–8) are an enzyme family that protects against oxidative damage. SODs are a class of enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide. In a case–control study, a decrease of GPx, SOD and catalase activity was detected in subjects with AD [44].

Preliminary study on PBMCs validated our findings on neuronal cells. In this ex-vivo system, administration of metformin caused a reduction in cell viability due to increased oxidative stress which was attenuated by the addition of insulin. This suggests that combination of these two substances may be beneficial. Many studies have suggested that interaction between the central nervous system (CNS) and systemic immune
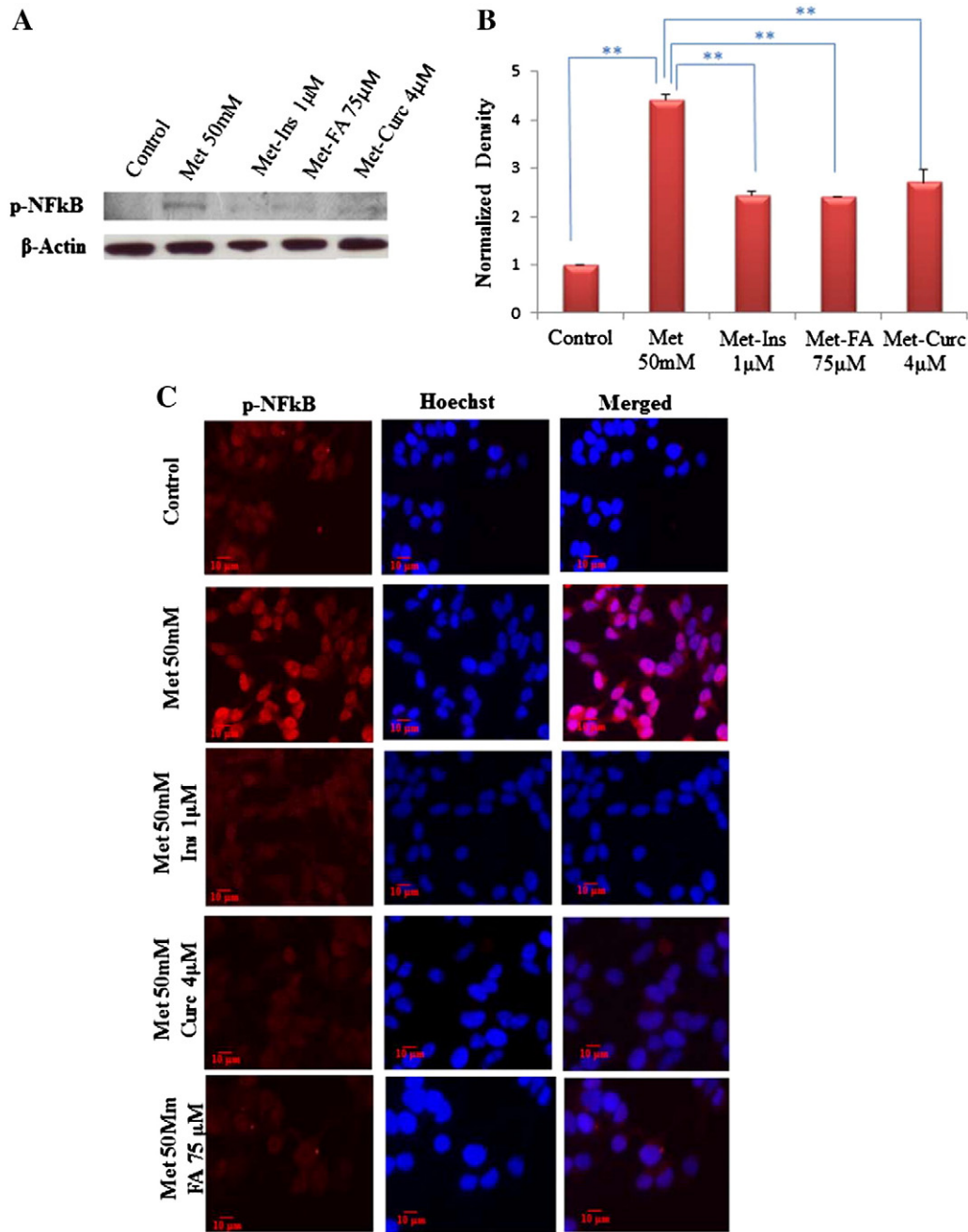
A

B

C

Fig. 8. Insulin and antioxidants inhibit the action of metformin on NF-κB activation. A) Western blot of LAN5 cells untreated (control), treated with metformin (Met 50 mM) alone, or with insulin (Met-Ins 1 μM), ferulic acid (Met-FA 75 μM) or curcumin (Met-Curc 4 μM) and incubated with anti-phospho-NF-κB (p-NFκB). Uniformity of gel loading was confirmed with β-actin as standard. B) Quantification of immunoreactivity was performed using densitometric analysis. **P < 0.02 versus indicated groups. C) Immunofluorescence of LAN5 untreated (control), or treated with metformin at 50 mM (Met) alone, with metformin and insulin at 1 μM (Met-Ins) with metformin and curcumin at 4 μM (Met-Curc) or with metformin and ferulic acid at 75 μM (Met-FA) and incubated with anti-phospho-NF-κB (p-NFκB) (red staining) and Hoechst 33258 (blue staining) then examined by fluorescent microscopy. Merged images of anti-phospho-NF-κB and Hoechst 33258 staining are shown. Bar, 10 μm.

responses may occur [45]. Neuroinflammation in particular is known to induce the translocation of CNS proteins, such as Aβ, or inflammatory mediators, across the blood–brain-barrier (BBB) which may lead to a systemic immune reaction and the recruitment of myeloid or lymphocytic cells into the CNS. Thus, the effect of drugs on lymphocytes may affect the integrity CNS behavior. Recently, multivariate analysis of cell cycle activity both in AD lymphocytes and an experimental model has demonstrated that disturbance occurs simultaneously in CNS and peripheral cells, suggesting that analysis of PMBCs may be useful in early diagnosis of AD [46,47].

Some experimental studies indicate that APP and presenilin expression can be regulated by the transcription factor NF-κB [23]. NF-κB is a

ubiquitous transcription factor activated by inflammation and oxidative, and other cellular stresses [48]. This activation results in a protective response aimed at restoring cellular homeostasis, which can become deleterious if activation is chronic. Several in vivo studies have suggested that Aβ production is dependent on NF-κB activation. In transgenic mice NF-κB activation has been shown to increase APP levels and BACE1 promoter and γ-secretase activities [49]. Furthermore, inhibition of NF-κB can reduce the plaque burden and improve the learning and memory deficits in mice. In contrast, in vitro studies in primary cultured neurons have demonstrated that Aβ may activate NF-κB by promoting nuclear translocation of its p50 and p65 subunits, suggesting the existence of a feedback control in which Aβ activates
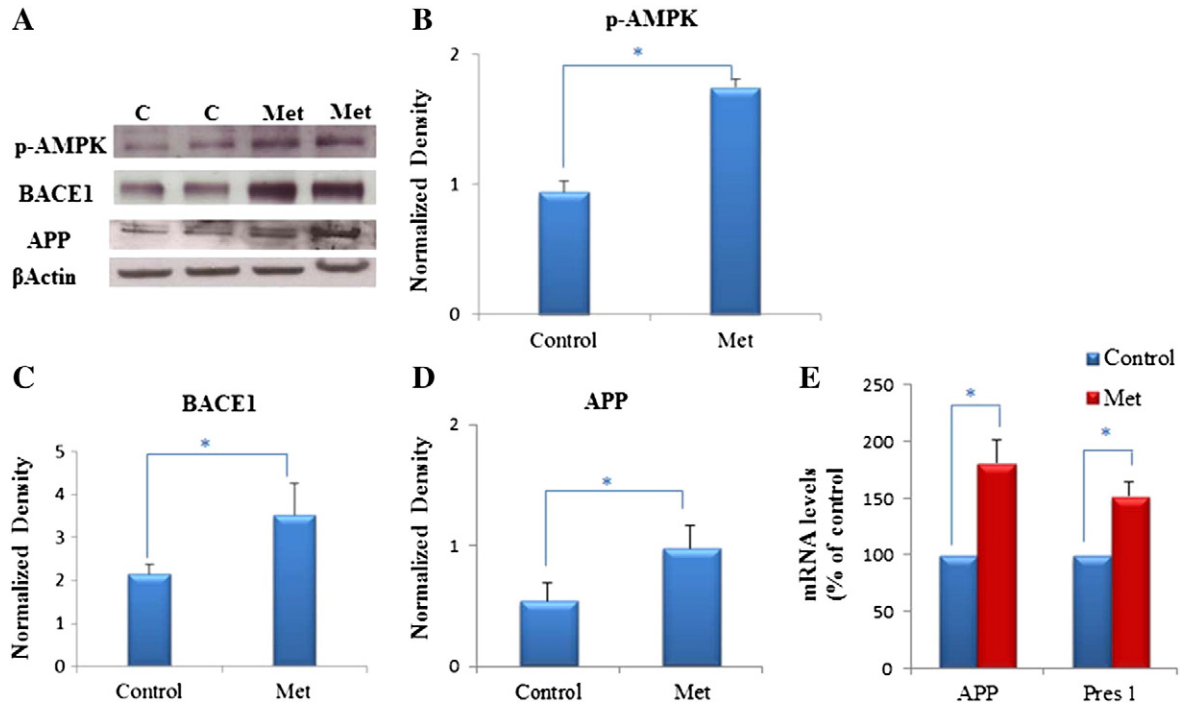
Fig. 9. Metformin activates AMPK, BACE1 and APP in mice. A) Western blot of brain lysates from C57BL6/J mice after drinking metformin (2 mg/ml) for 7 days and incubated with anti-pAMPK, anti-BACE1 and anti-APP. Uniformity of gel loading was confirmed with β-actin utilized as standard. B, C, D) Quantification of immunoreactivity was performed using densitometric analysis. E) Effect of metformin on APP and presenilin 1 (Pres-1) mRNA levels. APP and presenilin 1 (Pres 1) transcript levels were determined by quantitative real-time PCR. n = 4 animals in each group. *P < 0.05, versus indicated groups.
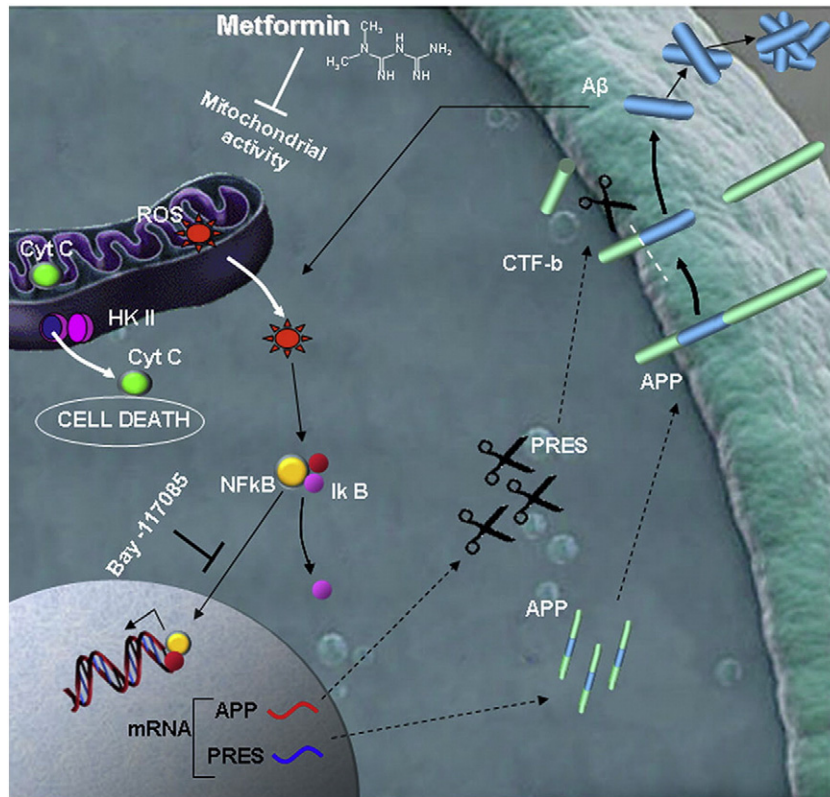

Fig. 10. A model to link metformin, Aβ production and cell degeneration. Exposure to metformin inhibits mitochondrial activity, induces ROS generation and decreases HK-II levels permitting perhaps PTP opening and cytochrome C (Cyt C) release, which lead to cell death. ROS production induces NFκB activation, which once dissociated from Ik B, translocates to the nucleus where it activates APP and presenilin 1 (PRES) gene transcription. Use of Bay11-7085 inhibits gene transcription induced by NFκB translocation. Increased APP and presenilin 1 levels lead to APP cleavage and Aβ production and aggregation. Aβ produces additional ROS that promote the translocation of NFκB to the nucleus, leading to the production of new APP and presenilin 1.

NF-κB, which, in turn, regulates the production of Aβ peptides [50]. Recently, experiments correlating NF-κB activation with APP expression, and with β- and γ-secretase expression, have demonstrated that, under physiological conditions, NF-κB triggers a repressive effect on Aβ-production, regulating Aβ-homeostasis, whereas under pathological conditions increases Aβ production [23]. Data reported here indicate that metformin contributes to NF-κB activation. In control cells, NF-κB is retained in the cytoplasm, presumably bound to its inhibitor Ik B. After exposure to metformin, NF-κB is activated and translocates into the nucleus where it initiates APP and presenilin transcription as demonstrated by blocking their transcription through Bay11-7085 inhibitor. However, even if our study does not establish whether NF-κB-associated APP and presenilin transcription is direct or involves intracellular intermediates, it is clear that metformin induced oxidative stress triggers NF-κB activation, which is inhibited by insulin and antioxidant molecules such as ferulic acid and curcumin.

Finally, we demonstrated that metformin is able to reach mouse brain as revealed by activation of AMPK one of the identified metformin molecular target, demonstrating the ability of the drug to cross the BBB.

The dose utilized for the treatment of the mice was deduced by those obtained by Chen et al. [11]. The authors found by the use of liquid chromatography in the same mouse strain (C57B6/J), that after 2 mg/ml administration of metformin its concentration in the plasma reach about 2 μM and in the brain about 1 μM, that results to be below the 10–40 μM reported in human plasma [14]. This dose was observed to up-regulates APP, BACE and presenilin in rodent brain, suggesting that this diabetes medication may cause severe side effects on accelerating AD progression.

We propose a model (Fig. 10) in which metformin induces ROS production, mitochondrial dysfunction, modulates HK-II expression potentially allowing opening of PTP and cytochrome C release, all of which lead to cell death. Cellular dysfunction is also promoted by up-regulation of ROS production genes and down-regulation of antioxidant genes. Induced oxidative stress activates NF-κB, which translocates to the nucleus where it triggers APP and presenilin gene expression. Increased APP and presenilin levels induce APP cleavage, Aβ production and aggregation. In a vicious circle Aβ, then cause the production of ROS which again promote the transfer of NF-κB to the nucleus.

5. Conclusions

Metformin induces an increase in the expression and processing of APP that is counteracted by insulin. Insulin also attenuates oxidative stress induced by metformin, as do antioxidants. Administration of both insulin and metformin recovers physiological conditions, suggesting a synergistic effect. The relationship identified between the key pathogenic peptide of AD and metformin/insulin is clearly of great interest and may have important implications for the treatment of T2DM and AD. However, large clinical trials are necessary to confirm and clarify the therapeutic efficacy of these compounds.

Conflict of interests

The authors declare no conflict of interests.

Acknowledgments

Transparency document

The Transparency document associated with this article can be found, in the online version.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2015.01.017.

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