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$CoCl_2$ Induces Apoptosis via the 18 kDa Translocator Protein in U118MG Human Glioblastoma Cells[†]

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ABSTRACT: The 18 kDa translocator protein (TSPO), formerly known as the peripheral-type benzodiazepine receptor, has been reported to be closely associated with the mitochondrial permeability transition pore (MPTP). TSPO is believed to exert pro-apoptotic functions via modulation of MPTP opening. Cobalt chloride ($CoCl_2$), which is sometimes used as a hypoxia mimicking agent, is also known to be able to induce apoptosis. One of our questions was whether $CoCl_2$ may induce apoptosis via the TSPO. To address this question, we used the U118MG human glioblastoma cell line. We applied the specific TSPO ligand, PK 11195, as well as TSPO knockdown with siRNA and studied their influence on the effects of $CoCl_2$ on cell death, including activation of the mitochondrial apoptosis pathway. To assay TSPO expression, we applied binding assays and Western blotting to whole cell homogenates and mitochondrial fractions. To assay activation of the mitochondrial apoptosis pathway, including some of the cellular mechanisms involved, we determined the incidence of collapse of the mitochondrial membrane potential ($\Delta\psi_m$) and cardiolipin oxidation and measured the level of DNA fragmentation to assay apoptotic rates. We found that the TSPO ligand, PK 11195, significantly counteracted induction of cell death by 0.4 mM $CoCl_2$, including apoptosis, collapse of the $\Delta\psi_m$, and cardiolipin oxidation. Moreover, we found that TSPO knockdown with siRNA fully protected against mentioned cell death mechanisms. Thus, we found that the TSPO is required for cell death induction by $CoCl_2$, including apoptosis. In conclusion, our studies show that activation of TSPO by $CoCl_2$ application is required for ROS generation, leading to cardiolipin oxidation, and collapse of the $\Delta\psi_m$, as induced by $CoCl_2$.

With this study, we address the question of whether the 18 kDa translocator protein (TSPO)[1] may be involved in pro-apoptotic effects of cobalt chloride ($CoCl_2$). $CoCl_2$ is sometimes used as a hypoxia mimicking agent. For example, application of $CoCl_2$ is known to interfere with heme formation (*1*). Furthermore, $CoCl_2$ may induce activation of various other mechanisms that react to changing oxygen levels, including hypoxia (*2*−*7*). For example, it was found that $CoCl_2$ induces apoptosis in PC12 cells, mediated both via the death receptors that are located in the plasma membrane and via the mitochondrial apoptosis pathway, including a regulatory role of the Bcl-2 family and activation of caspases 3, 8, and 9. The TSPO, also known as the peripheral-type benzodiazepine receptor (PBR), plays a role in various cellular functions (*8*−*10*). For example, it is well-known that the TSPO plays a role in activation of the mitochondrial apoptosis pathway (*10*−*15*). It is also known that the TSPO is closely associated with the 32 kDa voltage-dependent anion channel (VDAC) and the 30 kDa adenine nucleotide transporter (ANT) (*8*, *16*). VDAC and ANT are considered to form the mitochondrial permeability transition pore (MPTP) (*16*−*18*). Exposure to apoptosis-indu-

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[1]Abbreviations: ABTS, 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid; AIF, apoptosis-inducing factor; ANT, 30 kDa adenine nucleotide transporter; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; COX, cytochrome oxidase; EDTA, ethylenediaminetetraacetic acid; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride; MIP, mitochondrial intermediate peptidase; MPTP, mitochondrial permeability transition pore; NAO, 10-*N*-nonylacridine orange; PAP7, PBR-associated protein 7; PBR, peripheral-type benzodiazepine receptor; PI, propidium iodide; pk10, protein of 10 kDa; PK 11195, 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinolinecarboxamide; PRAX-1, PBR-associated protein 1; ROS, reactive oxygen species; Scr, scrambled siRNA with no homology to any mammalian sequence, used as a negative control for assays of TSPO siRNA knockdown cells; SDS, sodium dodecyl sulfate; siRNA, short interfering RNA; TSPO, 18 kDa translocator protein; VDAC, 32 kDa voltage-dependent anion channel; $\Delta\psi_m$, mitochondrial membrane potential.

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cing agents may result in opening of the MPTP, collapse of the mitochondrial membrane potential ($\Delta\psi_m$), increased permeability of the outer mitochondrial membrane, and release of apoptogenic factors into the cytosol (*12*, *19*, *20*). A dual role for reactive oxygen species (ROS) in apoptotic processes has been proposed, either as a cause for MPTP opening or as a byproduct of MPTP opening (*20*). Since TSPO can be intimately connected to VDAC and ANT, the TSPO is thought to exert pro-apoptotic functions via modulation of MPTP opening (*12*, *14*). In particular, activation of the TSPO leads to ROS generation, including cardiolipin oxidation (*12*). The oxidation of cardiolipins is suggested to cause detachment of cytochrome *c* from cardiolipins as well as activation of the VDAC by ROS (*12*, *21*). These processes then lead to the release of cytochrome *c* from the mitochondria and initiation of the mitochondrial apoptosis pathway (*12*, *14*, *15*). $CoCl_2$ can also induce ROS generation that may affect MPTP opening, potentially leading to apoptosis (*3*, *4*, *6*, *22*). With regard to a potential role of the TSPO in the hypoxia mimicking mechanisms of $CoCl_2$, it is also interesting to note that several papers have suggested that the TSPO can act as an oxygen sensor (*10*, *23*, *24*).

The purpose of this study is to determine potential interactions between $CoCl_2$ and TSPO in activation of the mitochondrial apoptosis pathway. Therefore, we applied the specific TSPO ligand, PK 11195, as well as TSPO knockdown with siRNA, and studied their influence on the effects of $CoCl_2$ on activation of the mitochondrial apoptosis pathway and cell death, including opening of the MPTP, ROS generation, and cardiolipin oxidation, in human U118MG glioblastoma cells. We chose the U118MG glioblastoma cell line since we are interested in brain cancer and its cell death-related mechanisms (*11*−*14*).

MATERIALS AND METHODS

Cell Culture. Cells of the human U118MG glioblastoma cell line were cultured as described previously (*14*).

Treatments. *(i) $CoCl_2$*. $CoCl_2$ at various concentrations (0.1−1 mM) was added to the cells for 24 h, as described for various cell lines in previous studies (*25*−*31*). After $CoCl_2$ incubation for 24 h, the cells were collected for the different assays mentioned below. Pilot studies by us showed that 0.4 mM $CoCl_2$ consistently resulted in significant levels of cell death of U118MG cells (*27*, *28*). This concentration is also comparable to those used by others on other cell lines (*29*−*31*).

(ii) PK 11195. We applied the TSPO ligand, PK 11195 (Sigma-Aldrich, Rehovot, Israel), at concentrations of 25, 50, 75, and 100 μM. The concentrations above 50 μM caused lethal effects in the cells comparable to what was found in previous studies (*10*, *14*). PK 11195 at concentrations of 25 and 50 μM did not show lethal effects, in accord with results of previous studies (*14*). To ensure we would not confound the results of PK 11195 application by its nonspecific lethal effects, we decided to use 25 μM PK 11195 for our experiments. The TSPO ligand, PK 11195 (25 μM), was added to the cells 24 h before the $CoCl_2$ and was also added on the day of the application of 0.4 mM $CoCl_2$. The vehicle for PK 11195 was 100% ethanol, of which 10 μL was added per 990 μL of culture medium (i.e., final ethanol concentration of 1%). For a control, the vehicle was added by itself (1% ethanol).

(iii) TSPO Knockdown with siRNA. TSPO knockdown with siRNA was conducted according to methods we described previously (*13*, *15*). For this study, we used a human TSPO sequence (20 bp) cloned into pSilencer 3.1 (H-1) hygro vector to produce cells with stable knockdown of TSPO. This vector was generously provided by G. Pasternak. This pSilencer hygro siRNA expression vector contains a hygromycin B resistance gene to enable antibiotic selection in mammalian cells. Scrambled siRNA with no homology to any mammalian sequence was used as a negative control (Scr), and this vector also contained the hygromycin B resistance gene. We conducted selection in 50 μg/ml hygromycin B for 2 weeks. We assessed TSPO expression with Western blot analysis and [^{3}H]PK 11195 binding analysis of the cells that survived the selection, to determine the effectiveness of the TSPO knockdown, as described below. Before each assay used for this study (cell viability, apoptosis, $\Delta\psi_m$, and ROS generation assays), we reassayed samples with Western blotting to verify the persistence of the TSPO knockdown.

TSPO Binding. Binding assays with the specific TSPO ligand, [^{3}H]PK 11195, were performed on homogenized U118MG cells, as described previously (*14*, *32*, *33*). Cells were seeded in six-well plates. On the day the cells became confluent, treatments were applied ($CoCl_2$, with or without PK 11195 application, with or without TSPO knockdown). After being treated for 24 h, the cells were scraped from the wells, collected together with their culture medium, and centrifuged (210*g* for 10 min at 4 °C). Following centrifugation, the cell pellets were snap-frozen in liquid nitrogen and stored at −70 °C until further use. On the day of the assay, the pellets were thawed and homogenized in 1 mL of phosphate-buffered saline (PBS) (Biological Industries, Beit Haemek, Israel), using a Kinematika Polytron (setting 6) for 10 s. Then protein content was determined by the method of Bradford (*34*). Binding studies were conducted using [^{3}H]PK 11195 (6 nM final concentration) as a radioligand (New England Nuclear, Boston, MA). After incubation for 90 min at 4 °C, the samples were vacuum-filtered through Whatman GF/C filters, washed three times with 4 mL of phosphate buffer, and placed in vials containing 4 mL of CytoScint (MP Biomedicals, Costa Mesa, CA). Radioactivity was counted after 12 h with a 1600CA Tri-Carb liquid scintillation analyzer (Packard, Meriden, CT), and specific binding was assessed as described previously (*32*).

Western Blot. Cells were seeded in six-well plates. On the day the cells became confluent, treatments were applied ($CoCl_2$, with or without PK 11195 application, with or without TSPO knockdown). After being treated for 24 h, cells were collected and prepared for Western blot analysis as described previously (*13*−*15*). Protein concentrations were determined according to the method of Bradford (*34*). β-Actin was used as a loading reference, as described previously (*14*, *15*). Human anti-TSPO antibodies (1:1000) prepared in our laboratory (*14*), monoclonal anti-VDAC antibodies (1:4000) obtained from Calbiochem (Merck, Darmstadt, Germany), and monoclonal anti-β-actin antibodies (1:15000) obtained from Sigma-Aldrich (Rehovot, Israel) were used. The secondary antibody was horseradish peroxidase-linked and directed against the host of the primary antiserum, i.e., either anti-rabbit IgG (1:3000 anti-TSPO) or anti-mouse IgG (1:4000 anti-β-actin) (GE Healthcare, Buckinghamshire, U.K.). Precision Protein Standards (Bio-Rad Laboratories, Hercules, CA) loaded on a separate lane on the gel were used as a reference for the MW of the proteins.

Mitochondrial Extraction. Cells were seeded in 75 cm^2 flasks and grown until they achieved confluency. Then they were collected for mitochondrial extraction and Western blot analysis. The cells were centrifuged (200*g* for 10 min); the pellet was

suspended in buffer B [5 mM Tris-HCl, 250 mM sucrose, and 1 mM EDTA (pH 7.5)], and protease inhibitor was added to the cells. After being incubated for 15 min, cells were gently homogenized with the aid of a pellet pestle motor (Kontes, Vineland, NJ) for 1 min and centrifuged at 600*g* for 10 min at 4 °C. The supernatant was collected and centrifuged (7000*g* for 12 min). After that, the pellet was collected and suspended in 200 μL of buffer B and protease inhibitor and centrifuged (8500*g* for 10 min). This suspension of the pellet and the centrifugation were performed twice. The resulting pellet was suspended in 100 μL of buffer B, protease inhibitor, and 2% SDS for incubation for 20 min at room temperature. After this incubation, the suspensions were centrifuged (10000*g* for 5 min) and the supernatant was collected. The solubilized suspensions were prepared for Western blots as described above. As it is well-known that TSPO can be present in monomeric as well as multimeric forms [with molecular masses of approximately 18, 36, 54, and 72 kDa (for reviews, see refs *9* and *10*)], we examined whether knockdown of TSPO affected the TSPO monomers and multimers equally in the mitochondrial fraction.

Cell Viability. To assay viability versus cell death, propidium iodide (PI) staining was preformed according to the instructions of the manufacturer (Molecular Probes, Carlsbad, CA). Cells were seeded in 24-well plates. On the day the cells became confluent, treatments were applied ($CoCl_2$, with or without PK 11195 application, with or without TSPO knockdown). After being treated for 24 h, the cells were trypsinized, collected, centrifuged at 210*g* for 10 min at 4 °C, and suspended in 0.5 mL of PBS solution containing 10 μg/mL PI purchased from Invitrogen (Carlsbad, CA). After incubation for 10 min at room temperature, routinely 30000 cells per sample were analyzed with the Calibur flow cytometer from Becton Dickinson (San Jose, CA) using CellQuest.

Mitochondrial Transmembrane Potential Analysis. JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine chloride) was used to assay changes in $\Delta\psi_m$, as described previously (*14*, *15*, *35*). As a positive control, the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was used. Cells were seeded in 24-well plates. On the day they became confluent, treatments were applied ($CoCl_2$, with or without PK 11195 application, with or without TSPO knockdown). After being treated for 24 h, the cells were trypsinized, collected from the wells, and centrifuged at 210*g* for 10 min at 4 °C. Cell pellets were resuspended in 0.5 mL of 5 μM JC-1 in PBS and incubated for 15 min at 37 °C. After incubation, the cells were centrifuged at 210*g* for 5 min and resuspended in 0.5 mL of PBS. Then, the cell suspensions were transferred to 5 mL of FALCON FACS tubes and analyzed with the flow cytometer.

DNA Fragmentation. The cell death detection ELISA^{PLUS} Kit (Cell Death Kit) (Roche Molecular Biochemicals, Mannheim, Germany) was used to determine relative levels of DNA fragmentation as an indication of apoptotic levels, as described previously (*11*, *14*, *15*). Cells were seeded in 24-well plates. On the day they became confluent, treatments were applied ($CoCl_2$, with or without PK 11195 application, with or without TSPO knockdown). After being treated for 24 h, the cells were trypsinized, collected from the wells, and centrifuged at 210*g* for 10 min at 4 °C. Cell pellets were lysed with lysis buffer according to the manual provided by the manufacturer. The lysate was centrifuged at 210*g* for 10 min. A fraction of the supernatant was transferred to the streptavidin-coated microtiter plate modules. Immunoreagent was added (anti-histone-biotin and anti-DNA-peroxidase in the incubation buffer), and after incubation with gentle shaking for 2 h, the modules were rinsed three times with incubation buffer. Then the signal for apoptosis (DNA fragments bound to the bottom of the wells of the streptavidin-coated microtiter plate) was labeled with a 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) solution, as provided with the Cell Death Kit, for 10 min. The signal for DNA fragmentation was labeled with an ABTS solution, as provided with the Cell Death Kit, for 10 min. The staining intensity by the ABTS solution was determined with an ELISA reader (Ceres UV 900, Bio-Tek, Burlington, VT), at a wavelength of 405 nm, using the reference wavelength of 490 nm. The ABTS solution by itself was used as a blank. The positive control was provided with the Cell Death Kit.

Cardiolipin Peroxidation Analysis. Cardiolipin in mitochondria contains a substantial proportion of highly unsaturated fatty acids and is therefore sensitive to oxidation. It is known that ROS can cause a decrease in cardiolipin content. 10-*N*-Nonylacridine orange (NAO) can bind specifically to nonoxidized cardiolipin phospholipids (*36*). A decrease in the cellular fluorescence of NAO is thought to reflect peroxidation or other modifications of cardiolipin. Therefore, we used NAO fluorescence measured with flow cytometry to assay cardiolipin peroxidation in mitochondria to determine the degree of oxidative injury to the mitochondria, as described previously (*12*). For this assay, cells were seeded in 24-well plates. On the day the cells became confluent, treatments were applied ($CoCl_2$, with or without PK 11195 application, with or without TSPO knockdown). After being treated for 24 h, the cells were trypsinized, collected from the wells, and centrifuged at 210*g* for 10 min at 4 °C. Cell pellets were resuspended in 0.5 mL of 10 μg/mL NAO in PBS and incubated for 30 min at 37 °C in the dark. Then the cell suspensions were transferred to 5 mL FALCON FACS tubes and analyzed with the flow cytometer.

Statistical Analysis. Results were expressed as means ± the standard deviation (SD) ($n > 5$). A Student's *t* test or a Mann−Whitney test was used for comparisons, as appropriate. As required, Kruskal−Wallis nonparametric analysis of variance followed by the Mann−Whitney test was used to analyze the data. Bartlett's test for homogeneity of variance was used to determine the appropriate model, i.e., parametric or nonparametric. Statistical significance is defined by $p < 0.05$.

RESULTS

Exposure of U118MG cells to various concentrations (0.1−1 mM) of $CoCl_2$ for 24 h showed concentration-dependent effects on cell death (Figure 1A). In particular, statistically significant lethal effects of $CoCl_2$ determined with PI appeared at a concentration as low as 0.3 mM (Figure 1A). Concentrations of 0.3−0.5 mM showed statistically significant increases in cell death levels of 10−25%, while concentrations up to and including 0.2 mM did not show such effects (Figure 1A). Concentrations of ≥0.6 mM caused 40% cell death and more of the total cell population, up to 90% with application of 1 mM $CoCl_2$ (Figure 1A). Furthermore, U118MG cells exposed to 1 mM $CoCl_2$ showed enhanced TSPO protein expression (5.2-fold) determined with Western blot analysis (Figure 1B,C). Binding assays showed similar results: an increase of 3.7-fold in the level of TSPO binding (data not shown). These increases in TSPO protein expression and binding were very variable. $CoCl_2$ at 1 mM also appeared to result in decreases in β-actin levels and increases in VDAC levels (Figure 1C). In contrast, U118MG cells exposed to 0.4 mM $CoCl_2$ showed a

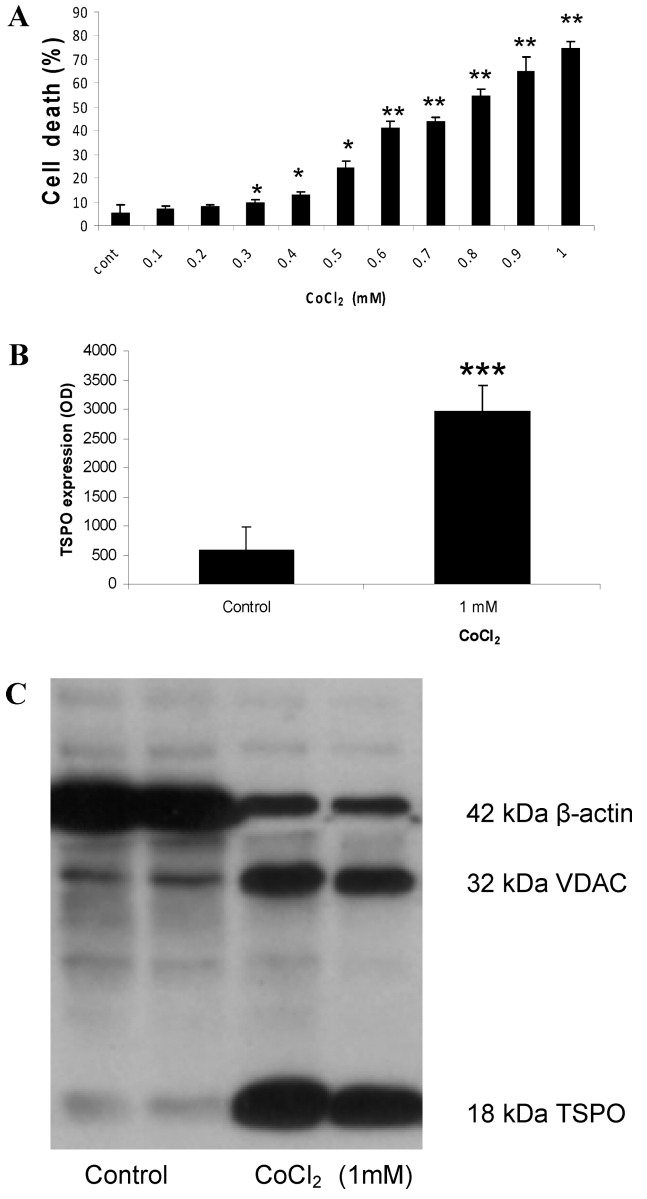

FIGURE 1: (A) Concentration-dependent effects of $CoCl_2$ treatment for 24 h on the viability of U118MG cells assayed by PI inclusion [n = 8 for control (cont), n = 5 for 0.1−1 mM $CoCl_2$, *p < 0.05 vs control, **p < 0.01 vs control]. (B) Treatment with $CoCl_2$ (1 mM, 24 h) caused an increase in the level of TSPO expression as assayed with Western blotting (n = 5, ***p < 0.001). OD is optical density as measured with the densitometer. (C) Representative example of a Western blot showing increases in the level of 18 kDa TSPO expression in U118MG cells exposed to 1 mM $CoCl_2$ for 24 h compared to vehicle-treated control cells. β-Actin and VDAC expression also appear to be affected by this treatment.

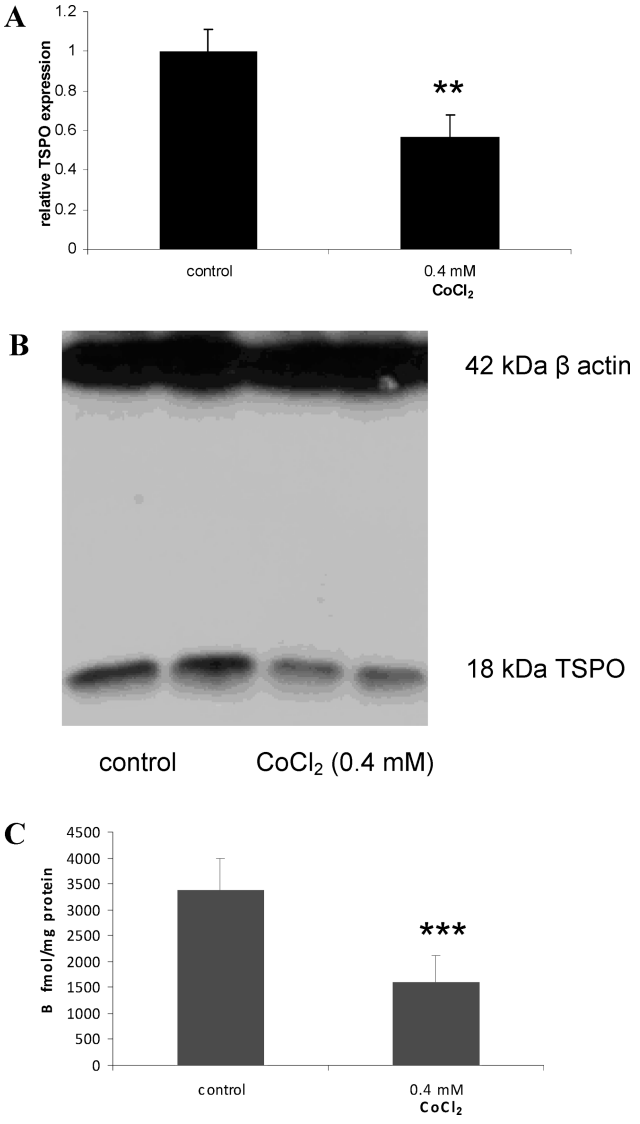

FIGURE 2: Treatment with $CoCl_2$ (0.4 mM, 24 h) caused a decrease in the level of TSPO expression. (A) Effect of $CoCl_2$ treatment on TSPO expression as determined by Western blotting, presented as densitometric measurements normalized to control (n = 5). (B) Representative Western blot of the level of TSPO expression reduced by $CoCl_2$. β-Actin does not appear to be affected by 0.4 mM $CoCl_2$. (C) Effect of $CoCl_2$ treatment on binding of the TSPO specific ligand, [^{3}H]PK 11195 (n = 6). Symbols and abbreviations: B, binding; 0.4 mM $CoCl_2$, U118MG cells treated for 24 h with 0.4 mM $CoCl_2$; control, U118MG cells treated with vehicle; **p < 0.01 vs control; ***p < 0.001 vs control.

significantly decreased level of TSPO protein expression (43%) determined with the aid of Western blotting (Figure 2A). With the 0.4 mM $CoCl_2$ exposure, β-actin levels were not affected (Figure 2B). The treatment of 0.4 mM $CoCl_2$ also caused an extremely significant decrease of 53% in the level of TSPO binding determined with a single concentration of the specific TSPO ligand, [^{3}H]PK 11195 (6 nM final concentration) (Figure 2C). As the effects of 0.4 mM $CoCl_2$ appeared to be very reproducible and specific and comparable to concentrations used in studies by others (see Materials and Methods), we chose a $CoCl_2$ concentration of 0.4 mM for our study (*27*, *28*).

Previous studies had shown that application of the classical TSPO ligands, Ro5 4864 and PK 11195, as well as knockdown of TSPO by genetic manipulation can protect various human and animal cell lines against apoptotic cell death (*10*−*15*). Therefore, we decided to determine whether such treatments also protected against the lethal effects of 0.4 mM $CoCl_2$ on human glioblastoma cell line U118MG. Applying PI uptake, we saw that the TSPO specific ligand, PK 11195, at 25 μM reduced levels of cell death otherwise caused by treatment for 24 h with 0.4 mM $CoCl_2$ (Figure 3A). In particular, this treatment with PK 11195 in combination with $CoCl_2$ showed a 54% reduction in the level of cell death in comparison to $CoCl_2$ treatment alone. In addition, this treatment with PK 11195 in combination with $CoCl_2$ resulted in a 48% reduction in apoptotic levels in comparison to $CoCl_2$ treatment alone (Figure 3B). Furthermore, 25 μM PK 11195 can reduce the incidence of $\Delta\psi_m$ collapse in cells treated for 24 h with 0.4 mM $CoCl_2$ (Figure 3C). In particular, this treatment with PK

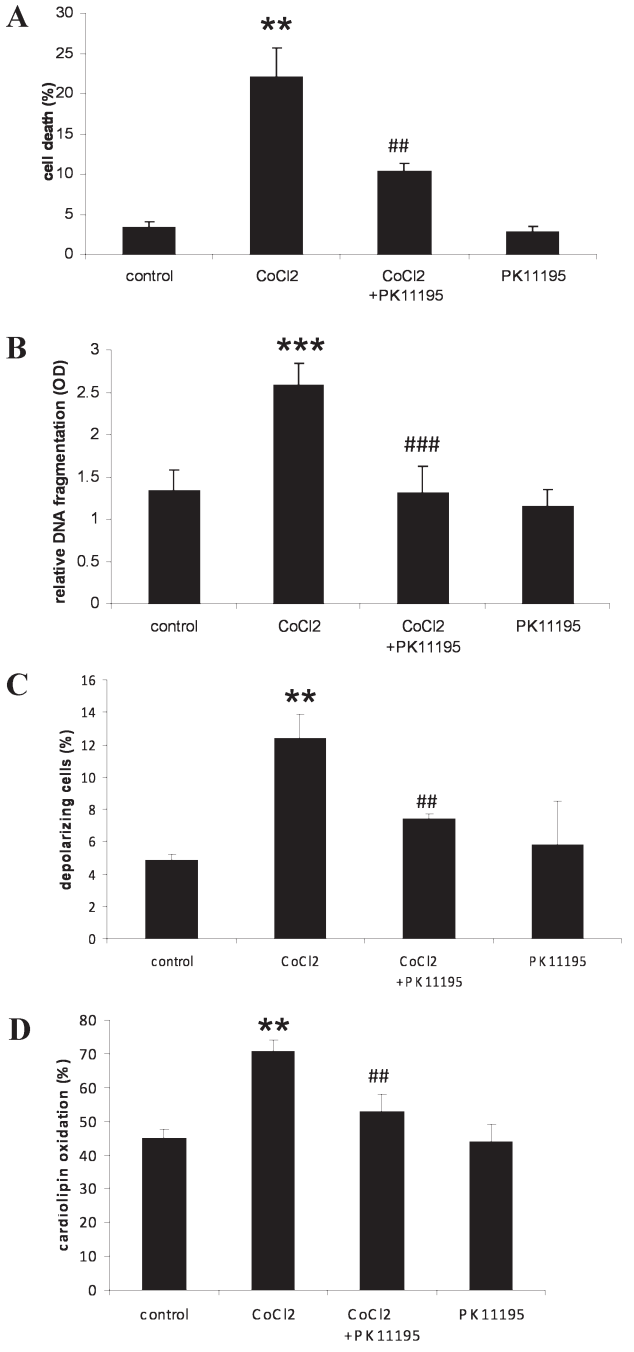

FIGURE 3: PK 11195 (25 μM) protects against (A) lethal effects, including (B) apoptosis, (C) collapse of $\Delta\psi_m$, and (D) cardiolipin oxidation due to $CoCl_2$ treatment (0.4 mM, 24 h) of U118MG cells. PK 11195 by itself had no effect on these parameters. (A) Percentage of dead cells determined with PI uptake ($n = 6$). (B) DNA fragmentation levels as an indicator for apoptosis determined with the Cell Death Kit ($n = 6$). (C) Incidence of $\Delta\psi_m$ collapse determined with JC-1 ($n = 6$). (D) Effects of PK 11195 on the incidence of cardiolipin oxidation determined with NAO uptake ($n = 6$). Symbols and abbreviations: $CoCl_2$, U118MG cells treated for 24 h with 0.4 mM $CoCl_2$; $CoCl_2$ + PK 11195, U118MG cells treated for 24 h with 0.4 mM $CoCl_2$ in combination with 25 μM PK 11195; PK 11195, cells treated for 24 h with 25 μM PK 11195; control, U118MG cells treated with vehicle; $^{**}p < 0.01$ vs control; $^{\#\#}p < 0.01$ vs cells treated with $CoCl_2$ only; $^{***}p < 0.001$ vs control; $^{\#\#\#}p < 0.01$ vs cells treated with $CoCl_2$ only.

11195 in combination with $CoCl_2$ showed a 42% reduction in the incidence of $\Delta\psi_m$ collapse in comparison to $CoCl_2$ treatment alone. We also found that 25 μM PK 11195 can enhance the level of NAO labeling as an indication of a reduction in cardiolipin oxidation in cells treated for 24 h with 0.4 mM $CoCl_2$ (Figure 3D). In particular, this treatment with PK 11195 in combination with $CoCl_2$ resulted in a 35% reduction in the numbers of cells showing a lack of NAO labeling in comparison to $CoCl_2$ treatment alone. Thus, PK 11195 appeared to reduce cardiolipin oxidation otherwise induced by $CoCl_2$. PK 11195 at 25 μM by itself had no effect on cell death, apoptosis, $\Delta\psi_m$, or cardiolipin oxidation (Figure 3 A−D).

Similar to PK 11195 treatments, TSPO knockdown counteracted the effects of exposure to $CoCl_2$. Moreover, the protective effects of TSPO knockdown were far more pronounced than of PK 11195 treatments. As described in Materials and Methods, we applied siRNA to induce TSPO knockdown. With this method, the level of TSPO expression as determined by Western blotting was reduced 2.4-fold (Figure 4A,B) and the level of TSPO binding was reduced 1.7-fold (Figure 4C). Applying Western blotting to mitochondrial extracts, we found that a 47% reduction of the level of 18 kDa TSPO had taken place in the mitochondria of siRNA cells in comparison to Scr control cells (Figure 4D−F). The Western blot analysis also suggested a reduction in the level of 36 kDa TSPO in siRNA knockdown cells (Figure 4D). The levels of higher-order TSPO multimers did not appear to be affected by our TSPO knockdown (Figure 4D). Mitochondrial VDAC levels also were not different between our siRNA TSPO knockdown cells and the Scr control cells (Figure 4E).

TSPO knockdown prevented cell death induced by treatment for 24 h with 0.4 mM $CoCl_2$ (Figure 5A). In particular, control (Scr) cells subjected to 0.4 mM $CoCl_2$ treatment showed 4.5-fold more cell death than Scr cells that were not treated with $CoCl_2$, while this $CoCl_2$ treatment did not enhance cell death levels in TSPO knockdown cells in comparison to otherwise untreated TSPO knockdown cells (Figure 5A). In addition, TSPO knockdown prevented apoptosis and collapse of the $\Delta\psi_m$ in cells treated for 24 h with 0.4 mM $CoCl_2$ (Figure 5B,C). In particular, Scr cells treated with $CoCl_2$ in this way exhibited 3.6-fold more apoptotic cell death than cells not treated with $CoCl_2$, while this $CoCl_2$ treatment did not enhance apoptotic cell death levels in TSPO knockdown cells (Figure 5B). With regard to $\Delta\psi_m$, Scr cells treated for 24 h with 0.4 mM $CoCl_2$ showed a 2.7-fold higher incidence of $\Delta\psi_m$ collapse than Scr cells not treated with $CoCl_2$, while this $CoCl_2$ treatment did not enhance the incidence of $\Delta\psi_m$ collapse in TSPO knockdown cells in comparison to untreated TSPO knockdown cells (Figure 5C). TSPO knockdown also appeared to reduce the level of cardiolipin oxidation in comparison to Scr cells (Figure 5D). In particular, Scr cells that were treated with 0.4 mM $CoCl_2$ exhibited 3.6-fold more cells with a reduction in cardiolipin NAO labeling than untreated Scr cells. In contrast, TSPO knockdown cells treated with $CoCl_2$ showed no sign of cardiolipin oxidation compared to the untreated knockdown cells. This indicates that ROS generation at mitochondrial levels by $CoCl_2$ is attenuated by TSPO knockdown.

DISCUSSION

This study demonstrates a new, potentially essential, apoptotic pathway for cell death triggered by $CoCl_2$. Previously, several other $CoCl_2$-related mechanisms have been reported. For example, $CoCl_2$ triggers transcriptional changes that mimic hypoxic responses, including upregulation of HIF1α, erythropoietin, and glycolytic enzymes (*4*, *6*). In addition, $CoCl_2$ can prevent the

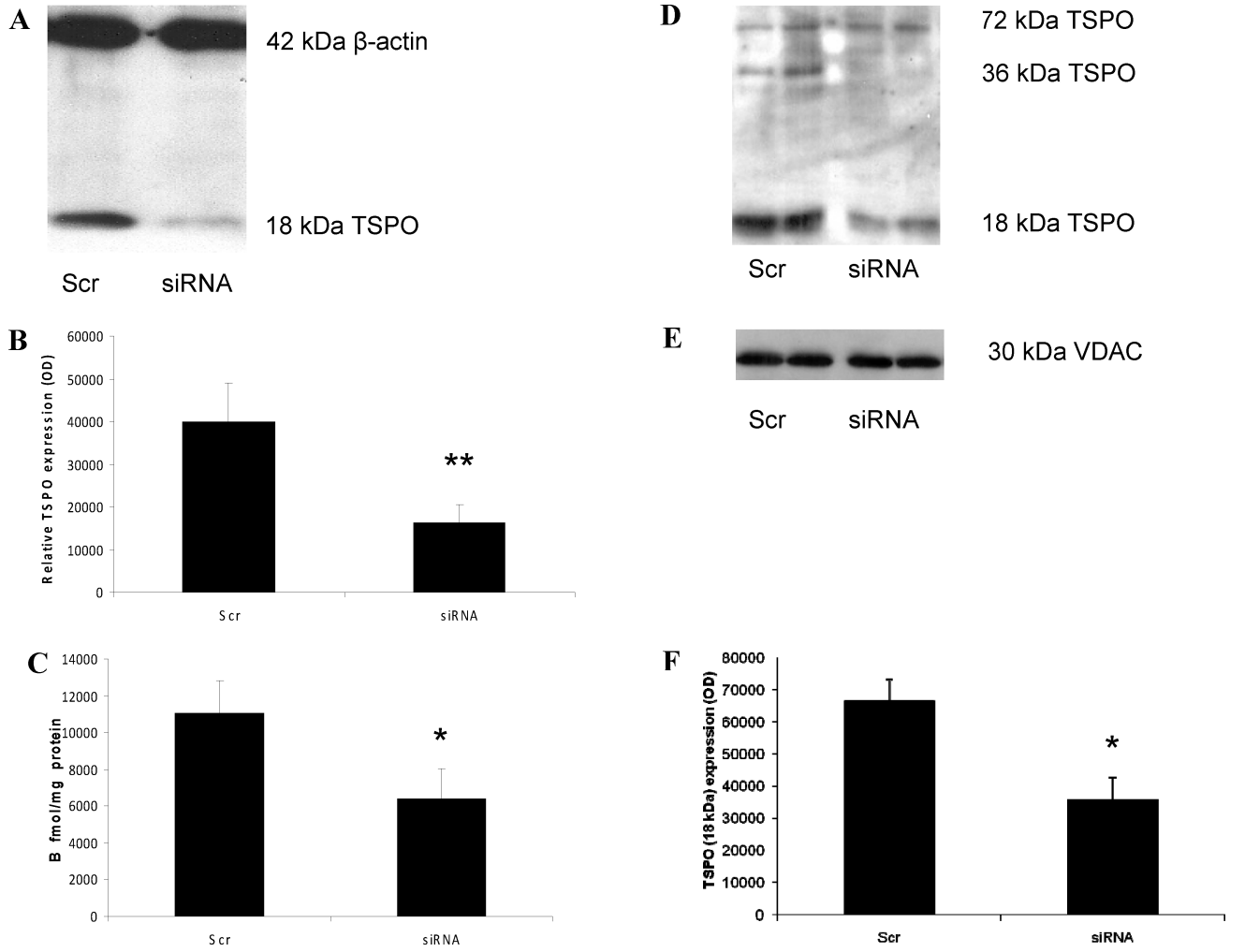

FIGURE 4: Stable transfection of U118MG cells with a vector expressing siRNA directed against TSPO reduces the level of TSPO expression (TSPO knockdown) compared to U118MG cells transfected with the same vector expressing a scrambled siRNA sequence (Scr), as determined with Western blotting (A and B) and [^{3}H]PK 11195 binding (C) in whole cell homogenates, and Western blot analysis of TSPO and VDAC in the mitochondrial extracts (D−F). (A) Representative example of a Western blot showing TSPO expression in Scr cells and TSPO knockdown cells. β-Actin is used as a loading control. (B) Densitometer analysis of Western blots shows a reduction in the level of TSPO protein expression in TSPO knockdown cells compared to Scr cells ($n = 6$). OD is optical density as measured with the densitometer. (C) The level of [^{3}H]PK 11195 binding (B, 6 nM) is reduced in TSPO knockdown compared to Scr cells ($n = 5$). **$p < 0.01$; *$p < 0.05$ vs Scr cells. (D) Representative example of a Western blot for TSPO expression in the mitochondria of Scr control cells and siRNA TSPO knockdown cells, showing reduction of the level of TSPO expression due to the siRNA knockdown. (E) VDAC expression was not affected by TSPO knockdown. (F) Densitometer analysis of Western blots showing a significant reduction of the level of 18 kDa TSPO protein expression in the mitochondria of siRNA TSPO knockdown cells compared to the mitochondria of Scr cells ($n = 4$). OD is optical density as measured with the densitometer. *$p < 0.05$ vs Scr cells.

normal processing of the precursor of cytochrome oxidase subunit 4 (COX4) and induces COX degradation very likely by inhibiting the mitochondrial intermediate peptidase (MIP) that cleaves the COX4 precursor protein (*7*). Generation of ROS during hypoxia as well as due to the presence of $CoCl_2$ has also been described (*4*, *30*, *37*, *38*).

In our study, applying siRNA knockdown of the TSPO, we found that $CoCl_2$ appears to require TSPO for the induction of cell death, including apoptosis, collapse of the $\Delta\psi_m$, ROS generation, and cardiolipin oxidation. Previous studies by us have shown that TSPO may be involved in ROS generation at mitochondrial levels causing cardiolipin oxidation, regulation of opening of the MPTP, including collapse of the $\Delta\psi_m$, and initiation of the mitochondrial apoptosis pathway (*10*−*15*). Loss of $\Delta\psi_m$ interferes with the production of ATP and may also lead to massive swelling of the mitochondrial matrix, potentially leading to rupture of the outer mitochondrial membrane (*38*−*41*). This may lead to cell death. Activation of the MPTP, including VDAC, without rupture of the mitochondrial membrane may also lead to apoptosis via release of apoptogenic factors, including cytochrome *c* and apoptosis-inducing factor (AIF) (*12*, *21*, *42*).

In this study, we found concentration-dependent induction of cell death by $CoCl_2$. In relation to this, we observed strong but very variable increases in the level of TSPO expression due to treatment with 1 mM $CoCl_2$. It has been reported previously that TSPO can contribute to induction of cell death, including apoptosis (*13*, *15*). We also expected to see smaller increases in the level of TSPO expression with a $CoCl_2$ concentration of 0.4 mM. To our surprise, this concentration resulted in a considerable decrease in the level of TSPO expression, while considerable cell death levels were still observed. Previous studies indicated that reduction in TSPO levels leads to reductions in cell death rates, including apoptosis (*10*−*15*). We suggest that the reduction in TSPO levels produced by 0.4 mM $CoCl_2$ may serve as a mechanism for counteracting cell death, otherwise caused by

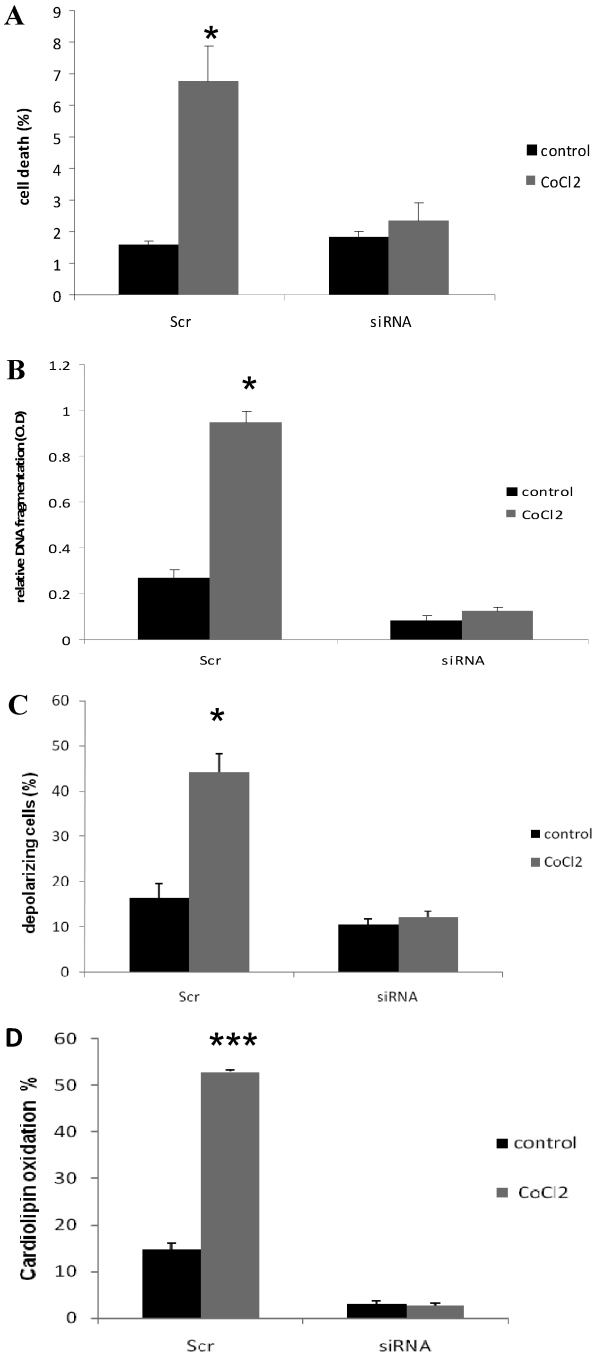

FIGURE 5: We found that TSPO knockdown with siRNA protects against (A) lethal effects, including (B) apoptosis, (C) collapse of $\Delta\psi_m$, and (D) cardiolipin oxidation due to of $CoCl_2$ treatment (0.4 mM, 24 h). (A) Percentage of dead cells determined with PI uptake ($n = 5$). (B) DNA fragmentation levels as an indicator for apoptosis determined with the Cell Death Kit ($n = 8$). (C) Incidence of $\Delta\psi_m$ collapse determined with JC-1 ($n = 5$). (D) Incidence of cardiolipin oxidation determined with NAO uptake ($n = 6$). Symbols and abbreviations: $CoCl_2$, U118MG cells treated for 24 h with 0.4 mM $CoCl_2$; control, U118MG cells treated with vehicle; Scr, U118MG cells stably transfected with a vector containing a scrambled DNA sequence; $^*p < 0.05$ vs control; $^{***}p < 0.001$ vs control.

$CoCl_2$. Obviously, the reduction in the level of TSPO expression in this paradigm (0.4 mM $CoCl_2$) did not fully protect against cell death. Either the lethal effects of $CoCl_2$ are too strong and/or bypass the TSPO, or the TSPO activity is not sufficiently reduced. To test the hypothesis that reductions in the level of TSPO expression or inhibition of TSPO functions would protect the U118MG cells against cell death induced by $CoCl_2$ in this study, we applied the TSPO specific ligand, PK 11195, and also TSPO knockdown with siRNA which both had shown inhibitory effects with regard to apoptosis in previous studies (*10*, *12*−*15*). Indeed, we found that TSPO knockdown in this study fully protected against cell death induced by 0.4 mM $CoCl_2$, including apoptosis, collapse of the $\Delta\psi_m$, and cardiolipin oxidation. Previous studies have shown that multimers of the TSPO play important roles in TSPO-related functions, such as transport of cholesterol over the outer mitochondrial membrane (*43*). In this study, the siRNA knockdown we applied appeared to reduce the level of 18 kDa TSPO expression in the mitochondria, suggesting that the mitochondrial 18 kDa TSPO plays a major role in cell death and apoptosis induction by $CoCl_2$. Thus, the 18 kDa TSPO appears to be essential for induction of cardiolipin oxidation, collapse of the $\Delta\psi_m$, and consequently cell death, including apoptosis by $CoCl_2$ in the paradigm we applied. Our study also suggests a reduction in 36 kDa levels due to the siRNA knockdown we applied, as seen with Western blot, suggesting that this TSPO dimer may also be involved in $CoCl_2$-induced apoptosis. In addition, reminiscent of TSPO knockdown with siRNA, PK 11195 application also protected against cell death induced by 0.4 mM $CoCl_2$, including apoptosis, collapse of the $\Delta\psi_m$, and cardiolipin oxidation, albeit providing less total protection than TSPO knockdown. This suggests that, as postulated, strong inhibition of TSPO like that which can be achieved with siRNA can provide full protection against cell death, including apoptosis and ROS generation induced by $CoCl_2$, while moderate inhibition, which can be achieved with PK 11195, can provide partial protection against cell death, including apoptosis and ROS generation. The TSPO-related mechanisms apparently induced by $CoCl_2$ in U118MG cells are presented in Figure 6.

As mentioned above, while 1 and 0.4 mM $CoCl_2$ applications both increased cell death levels, the 1 mM $CoCl_2$ application increased the level of TSPO expression, but the 0.4 mM $CoCl_2$ application decreased the level of TSPO expression. Studying and reporting such observations in isolation could lead to radically different, if not opposing, interpretations of the involvement of TSPO in functions such as initiation of the mitochondrial apoptosis pathway. Such opposing viewpoints indeed have been reported, as recognized by several researchers (e.g., refs *10*, *15*, and *44*−*46*). Our report, presenting radical differences in effects on TSPO expression by 0.4 mM $CoCl_2$ and 1 mM $CoCl_2$, emphasizes the need to exercise great care in the interpretation of results and not to neglect alternative interpretations from the rapidly expanding body of observations relating to the TSPO. $CoCl_2$ concentrations of 0.1−1 mM are often used to mimic hypoxia (*47*, *48*). It also has been shown that concentrations of $CoCl_2$ lower than 0.5 mM typically are effective in mimicking hypoxia effects (*29*−*31*). This supports our conclusion that 0.4 mM $CoCl_2$ is an adequate concentration for studying $CoCl_2$-related effects in human U118MG glioblastoma cells. Moreover, the various changes in β-actin and VDAC expression after 1 mM $CoCl_2$ treatment indicated effects on other proteins in addition to effects on TSPO expression that may potentially complicate the interpretation of the results. Such complications did not appear to occur with 0.4 mM $CoCl_2$, further indicating that 0.4 mM $CoCl_2$ was an appropriate concentration for attaining straightforward answers regarding the involvement of the TSPO in cell death and apoptosis induction by $CoCl_2$.

To postulate an interpretation regarding the results of our study, we suggest that the relatively high concentration of $CoCl_2$

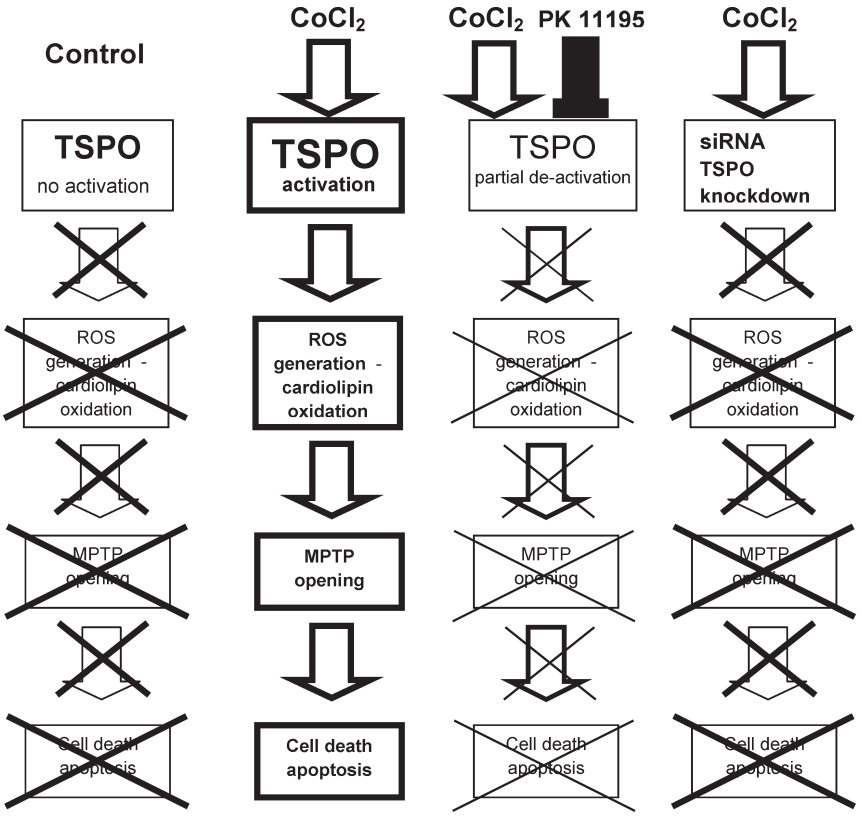

FIGURE 6: Flow diagrams showing the effects of siRNA TSPO knockdown and PK 11195 treatment on induction of cell death by $CoCl_2$ exposure in U118MG human glioblastoma cells. As presented in the flow diagram with the heading $CoCl_2$, our study showed that $CoCl_2$ activation of the TSPO causes ROS generation and cardiolipin oxidation, leading to opening of the MPTP and, consequently, cell death, including apoptosis. These processes typically are not activated in control cells, as presented in the flow diagram with the heading Control. The diagram with the heading $CoCl_2$ illustrates that knockdown of TSPO expression by siRNA prevents this sequence of events leading to cell death otherwise induced by $CoCl_2$. Similarly, as shown in the flow diagram with the heading and PK 11195, inhibition of TSPO activation by the TSPO ligand, PK 11195, also inhibits this sequence of events leading to cell death, albeit less effectively than TSPO knockdown by siRNA. The thick crosses indicate a lack of activation, and thin crosses indicate a reduced level of activation. Our study suggests that $CoCl_2$ activates TSPO, as indicated with TSPO knockdown by siRNA, while PK11195 reduces the level of this activation of the TSPO. The large empty arrows indicate activation. The large filled pipe indicates inhibition.

(1 mM) may have a general toxic effect on many intracellular mechanisms, irrevocably leading to cell death. This may also be reflected by our limited observations of changes not only in VDAC but also in β-actin expression. We assume that the increase in the level of TSPO expression under these conditions contributes to the high level of cell death. The less severe effect of 0.4 mM $CoCl_2$ may include self-protecting mechanisms of the cells, one of which may be reduction of the level of TSPO expression. The less severe effect of 0.4 mM $CoCl_2$ (compared to 1 mM $CoCl_2$) may also be reflected by the unaffected β-actin levels we observed. In other words, high levels of stress may induce activation of the mitochondrial apoptosis pathway via enhanced TSPO expression, while relatively low levels of stress may reduce the level of TSPO expression to restrict activation of the mitochondrial apoptosis pathway.

As $CoCl_2$ is considered to be a hypoxia mimicking agent, our results may have implications for our understanding regarding the involvement of TSPO in conditions such as ischemia, stroke, asphyxia, and limited oxygen supply to solid tumors, including glioblastoma, a form of cancer which is the subject in which we are interested (*11*−*14*). For example, cobalt is known to interfere in the production of heme from protoporphyrin IX (*1*, *49*). In this context, it is interesting to note that protoporphyrin IX is a high-affinity endogenous TSPO ligand. Interactions of protoporphyrin IX with the TSPO are considered to be part of the conversion of protoporphyrin IX to heme (*50*, *51*).

To the best of our knowledge, this is the first study showing that $CoCl_2$ can induce cell death, including apoptosis, via activation of the TSPO. The fact that knockdown of the TSPO induced by siRNA prevents cell death, including apoptosis, otherwise caused by $CoCl_2$, testifies to a role of the TSPO in these processes of cell death. Some key mechanisms related to the TSPO, including apoptosis, are summarized in Figure 6. It would be interesting to expand this study to other cell types which may suggest whether the effects we found in the cells of our interest may also be true for other systems. In such systems, one could further explore the question of the involvement of the TSPO monomer and multimer in cell death induced by $CoCl_2$. As $CoCl_2$-induced cell death appears to require TSPO activation, the model presented here may serve to in general test TSPO ligands for their efficacy. As our data suggest that the MPTP pore is involved in TSPO-related functions, further studies may be designed to target other compartments of the MPTP. The VDAC and the ANT are considered to be the core of the MPTP, while several other proteins, such as Bcl-2, hexodinase, creatine kinase, PBR-associated protein 7 (PAP7), protein of 10 kDa (pk10), and PBR-associated protein 1 (PRAX-1), are also closely associated with the MPTP (for reviews, see for example refs *9*, *10*, and *18*). Other questions may address the mechanisms by which $CoCl_2$ affects TSPO expression and function. This may, for example, include enhancement of TSPO mRNA levels or enhancement of TSPO mRNA translation (*52*−*54*).

In conclusion, the TSPO appears to be part of the cellular mechanisms involved in cell death induced by $CoCl_2$. The mechanisms shown to be involved in this study are TSPO expression levels, ROS generation, cardiolipin oxidation, collapse of the $\Delta\psi_m$, and cell death, including apoptosis. In particular, our knockdown experiments showed that TSPO is required for induction of cell death and apoptosis induced by $CoCl_2$.

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