The mitochondrial respiratory complex I is a target for 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 action

B. Martínez,* A. Pérez-Castillo,[1,†] and A. Santos[1,*]

Departamento de Bioquímica y Biología Molecular,* Facultad de Medicina, Universidad Complutense de Madrid, Madrid, Spain; and Instituto de Investigaciones Biomédicas,† Consejo Superior de Investigaciones Cientificas-Universidad Autonoma de Madrid, Madrid, Spain

Abstract The prostaglandin J_2 derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a very active compound with important effects on inflammation, apoptosis, and cell growth processes. To exert this broad range of effects, 15d-PGJ_2 binds and alters the activity of diverse proteins, which consequently are postulated to be mediators of its action. Among them are the transcription factors peroxisome proliferator-activated receptor γ and nuclear factor κB, which are thought to play an essential role in the antitumorigenic and anti-inflammatory actions of 15d-PGJ_2. Here, we show that 15d-PGJ_2, at micromolar concentrations, efficiently blocks state 3 oxygen consumption in intact nonsynaptic mitochondria isolated from rat cerebral cortex. This effect is attributable to the inhibition by this prostaglandin of the activity of the enzyme NADH-ubiquinone reductase (complex I) of the mitochondrial respiratory chain. In addition to this, 15d-PGJ_2 dramatically increases the rate of reactive oxygen species generation by complex I. The inhibition by 15d-PGJ_2 of complex I activity was abolished by dithiothreitol, which raises the possibility that adduct formation with a critical component of complex I accounts for the inhibitory effect of this prostaglandin. These results clearly identified mitochondrial complex I as a new target for 15d-PGJ_2 actions.—Martínez, B., A. Pérez-Castillo, and A. Santos. **The mitochondrial respiratory complex I is a target for 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 action. *J. Lipid Res.* 2005.** 46: **736–743.**

Supplementary key words respiratory chain • reactive oxygen species • cyclopentenone prostaglandins

Prostaglandin J_2 (PGJ_2) and its metabolites Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2) are naturally occurring derivatives of prostaglandin D_2, the most abundant prostaglandin in normal tissues (1). They have been shown to exert important effects on diverse biological processes, such as inflammation, cell growth, and apoptosis, with 15d-PGJ_2 as the most active component of this group of cyclopentenone prostaglandins (2, 3).

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Multiple mechanisms have been proposed to explain the diversity of action of 15d-PGJ_2, although the relative importance of each of them is not completely established. 15d-PGJ_2 is the most active natural ligand of the peroxisome proliferator-activated receptor γ (PPARγ) (4, 5). PPARγ is a transcription factor that belongs to the superfamily of nuclear receptors and therefore regulates gene expression in a ligand-dependent manner (6, 7). In addition to 15d-PGJ_2, many other compounds have been shown to activate PPARγ. Among them are the antidiabetic drugs thiazolidinedione and the nonsteroidal anti-inflammatory drugs (2). It is believed that PPARγ mediates, at least in part, the actions of 15d-PGJ_2 on cell growth and apoptosis through the regulation by this transcription factor of the expression of genes critical to these processes, such as cyclin D1 and D2 (8, 9), cyclin-kinase inhibitors $p21^{warf1/cip1}$ and $p27^{kip1}$ (8, 10), tumor suppressors phosphatase and tensisn homologue deleted from chromosoma 10 (PTEN) and breast cancer susceptibility gene 1 (BRCA1) (11, 12), and bcl2 family members (13, 14).

The transcription factor nuclear factor κB (NF-κB) is another important target for 15d-PGJ_2 action. In the unstimulated state, NF-κB is sequestered in the cytosol by the repressor protein inhibitor κB (I-κB) and consequently inactive. Upon cellular signaling, I-κB is phosphorylated by I-κB kinase, resulting in the degradation of I-κB and the translocation of NF-κB to the nucleus, where it regulates, among other functions, the expression of genes implicated in inflammatory processes (15). 15d-PGJ_2 inhibits NF-κB action by blocking I-κB kinase activity and NF-κB binding to DNA (16, 17). NF-κB inhibition by 15d-PGJ_2 is thought to represent the major pathway in the anti-inflammatory effects of this prostaglandin. In addition to PPARγ

Abbreviations: DCFH, dichlorofluorescin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; I-κB, inhibitor κB; NF-κB, nuclear factor κB; PGJ_2, prostaglandin J_2; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species.

[1] To whom correspondence should be addressed.
e-mail: piedras3@med.ucm.es (A.S.);
aperez@iib.uam.es (A.P-C.)

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and NF-κB, other transcription factors, signal transduction proteins, and membrane receptors have been shown to be targeted by 15d-PGJ_2 (18–21).

Finally, it has been also shown that 15d-PGJ_2 increases the intracellular production of reactive oxygen species (ROS), which is considered to be relevant for the induction of apoptosis and the activation of stress-activated and mitogen-activated protein kinases (22–27).

Here, we show that 15d-PGJ_2 is a potent inhibitor of mitochondrial respiration and that this inhibition is accomplished by efficiently blocking the activity of respiratory complex I. Consequently, oxygen consumption in respiratory state 3 is inhibited only in the presence of NADH-generating substrates, but not in the presence of succinate plus rotenone. In addition to this, 15d-PGJ_2 dramatically increases the rate of ROS production. Altogether, these results identify mitochondrial respiratory complex I as a new target for 15d-PGJ_2 action, which could be important for better understanding the biological effects of this prostaglandin.

EXPERIMENTAL PROCEDURES

Materials

Wistar rats were from our own breeding colony, and all experiments were conducted in accordance with the Spanish Guidelines for the Care and Use of Laboratory Animals. 15d-PGJ_2 and rosiglitazone were from Calbiochem (San Diego, CA). Prostaglandins A_2, D_2 and J_2, NADH, rotenone, and horseradish peroxidase were from Sigma (St. Louis, MO). Percoll was from Amersham Pharmacia Biotech (Uppsala, Sweden). Dichlorofluorescin (DCFH) diacetate was from Molecular Probes (Eugene, OR).

Mitochondria isolation

Rat cerebral cortex nonsynaptic mitochondria were isolated according to the method described by Dunkley et al. (28). Briefly, rat cerebral cortex was dissected from the brains of Wistar rats (100–150 g), and 1g of tissue was homogenized in 10 ml of ice-cold medium containing 0.32 M sucrose, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.4, in a Teflon-glass homogenizer and centrifuged at 1,000 *g* for 5 min. The supernatant was saved and centrifuged at 9,500 *g* for 10 min. The pellet was resuspended in 3 ml of a medium containing 0.32 M sucrose, 1 mM EDTA, 0.25 mM DTT, and 10 mM Tris-HCl, pH 7.4, then centrifuged in a discontinuous Percoll gradient (23, 10, and 3%) at 22,000 *g* for 10 min. The fraction enriched in nonsynaptic mitochondria was resuspended and washed in the homogenization buffer and immediately used for oxygen consumption and ROS generation or frozen at −80°C until used for the determination of complex I activity. All of the preparative steps were performed at 0–4°C. Protein content was measured by Bradford's procedure (29) using BSA as a standard.

Determination of the rate of oxygen consumption and hydrogen peroxide production

Oxygen consumption in freshly prepared mitochondria was measured polarographically with a Clark-type electrode (Oxigraph Hansatech) at 30°C with constant stirring. Mitochondria (0.7 mg of protein) were suspended in 1 ml of respiratory medium containing 75 mM sucrose, 50 mM KCl, 5 mM KH_2PO_4, 0.5 mM EDTA, 5 mM $MgCl_2$, and 30 mM Tris-HCl, pH 7.4. The basal rate of respiration (state 4) was determined in the presence of diverse substrates: glutamate/malate (5 mM each), pyruvate (5 mM), or succinate (5 mM). When succinate was the substrate, 5 μM rotenone was also added. The active state of respiration (state 3) was initiated by the addition of 0.5 mM ADP.

Mitochondrial generation of ROS was determined in freshly prepared mitochondria by measuring hydrogen peroxide-dependent oxidation of DCFH in the presence of horseradish peroxidase. For that purpose, DCFH was obtained from the stable reagent DCFH diacetate by alkaline treatment (30). Mitochondria (0.1 mg of protein) were incubated with 1.5 μM DCFH at 22°C in 1 ml of medium containing 75 mM sucrose, 5 mM K_2HPO_4, 40 mM KCl, 3 mM $MgCl_2$, 0.5 mM EDTA, 30 mM Tris-HCl, pH 7.4, and 0.5 μM horseradish peroxidase, and the rate of hydrogen peroxide production was estimated by measuring the linear fluorescence increase (excitation at 475 nm, emission at 525 nm) in a Perkin-Elmer 650 fluorimeter.

Complex I activity

The enzymatic activity of NADH decylubiquinone reductase was spectrophotometrically assayed by following the decrease in absorbance at 340 nm resulting from the oxidation of NADH as previously described (31). Briefly, 50 μg of mitochondrial protein was resuspended in 1 ml of medium containing 20 mM KH_2PO_4, 8 mM $MgCl_2$, 1 mM KCN, and 2.5 mg/ml BSA, pH 7.2, in the presence of 150 μM NADH and incubated at 30°C until the optical density value was stabilized. The reaction was then started by the addition of decylubiquinone at a final concentration of 50 μM. In parallel, NADH decylubiquinone reductase activity was determined in the presence of 10 μM rotenone.

RESULTS

Oxygen consumption

Oxygen consumption was determined in nonsynaptic mitochondria isolated from rat cerebral cortex, as indicated in Experimental Procedures. As shown in **Fig. 1**, no significant effect of 15d-PGJ_2 was observed on oxygen consumption in respiratory state 4 at concentrations as high as 30 μM. In addition, no effect of 15d-PGJ_2 (10 μM) on oxygen consumption was observed in low-energy respiratory states 1 and 2 (data not shown), suggesting that this compound has no uncoupling effect. When respiratory state 3 was induced by the addition of ADP at a final concentration of 0.5 mM, a significant increase in oxygen consumption was observed. The increase was 3.5-fold for glutamate/malate, 3.2-fold for pyruvate, and 2.7-fold for succinate, clearly indicating that the mitochondria used in these studies are functional. This increase was blocked by the presence of 15d-PGJ_2 when the substrate was glutamate/malate; therefore, at a concentration 5 μM, only a 2-fold increase in oxygen consumption was observed after the addition of ADP (Fig. 1A). The same inhibitory effect by this prostaglandin was observed when pyruvate, another NADH-generating substrate, was used. In contrast, no effect of 15d-PGJ_2 on mitochondrial respiration was observed when the substrate was succinate in the presence of rotenone. A 2.7-fold induction in oxygen consumption was observed after the addition of ADP in both the presence and absence of 10 μM 15d-PGJ_2 (Fig. 1B). Succinate is oxidized by respiratory complex II; therefore, these re-

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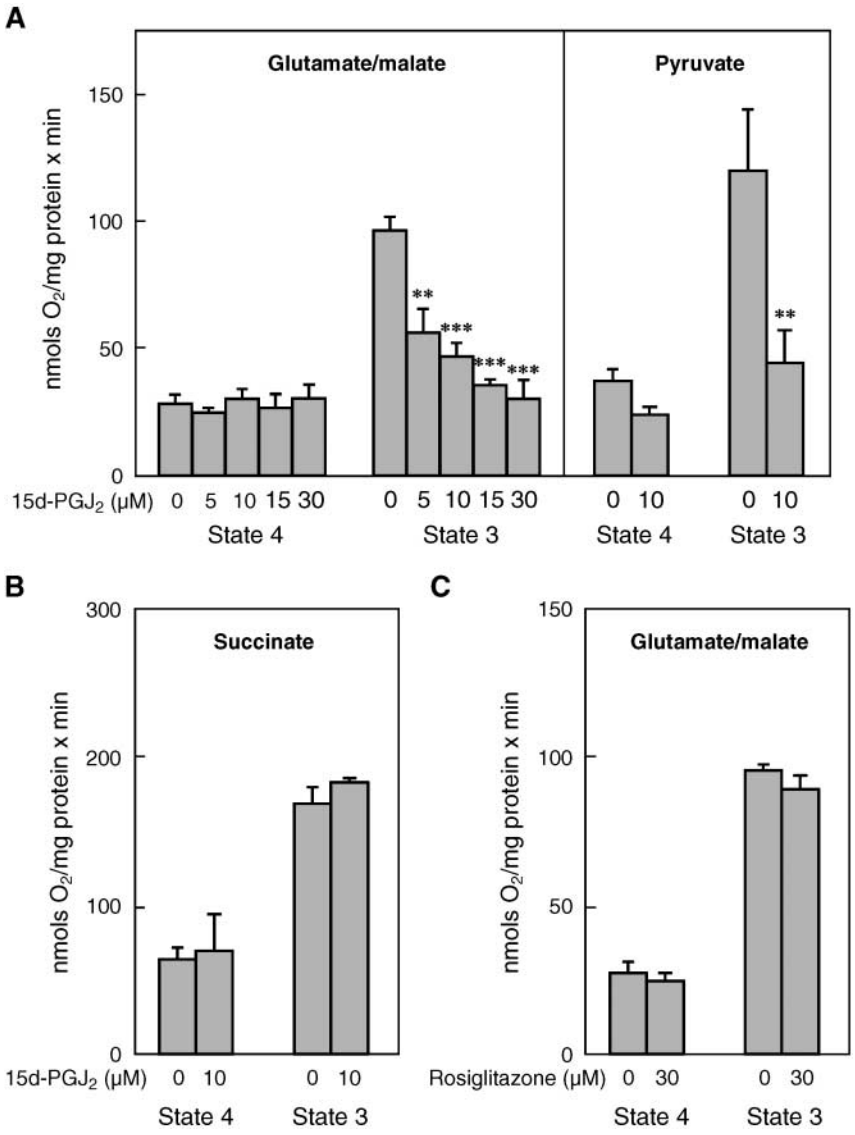

Fig. 1. Effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on mitochondrial respiration. Nonsynaptic mitochondria were isolated from rat cerebral cortex, and the rate of oxygen consumption was determined as indicated in Experimental Procedures. A: Effect of different concentrations of 15d-PGJ_2 on oxygen consumption in respiratory states 4 and 3 using the NADH-generating substrates glutamate/malate and pyruvate. B: Effect of 15d-PGJ_2 on oxygen consumption in the presence of succinate as substrate. C: Effect of rosiglitazone on oxygen consumption using glutamate/malate as substrate. The values shown represent means of at least five different experiments in duplicate, and the bars represent standard errors of the mean. ** $P \leq 0.01$ and *** $P \leq 0.001$ versus state 3 oxygen consumption in the absence of 15d-PGJ_2.

sults suggest that complex I is the target of 15d-PGJ_2 action on mitochondrial respiration.

Because 15d-PGJ_2 is a potent activator of the nuclear receptor PPARγ and the presence of these receptors has been described also within the mitochondrial matrix of certain tissues (32), we decided to test the action of rosiglitazone, a TZD that binds this nuclear receptor with high affinity, on mitochondrial respiration. As shown in Fig. 1C, no inhibition of complex I activity was observed with 30 μM rosiglitazone, a concentration known to efficiently activate PPARγ (12). Therefore, these results suggest that 15d-PGJ_2 action in the mitochondria is PPARγ-independent.

Mitochondrial complex I activity

The activity of NADH-ubiquinone reductase complex I was enzymatically determined in nonsynaptic mitochondria isolated from rat cerebral cortex, as described in Experimental Procedures, and the effect of 15d-PGJ_2 was analyzed. As shown in **Fig. 2A**, 15d-PGJ_2 blocks complex I activity; therefore, at a concentration of 5 μM, this prostaglandin inhibits 50% of the enzymatic activity of this complex. In contrast, no effect was observed in the presence of 30 μM rosiglitazone (Fig. 2B), a well-known PPARγ agonist. These results are clearly in agreement with those describing the effect of 15d-PGJ_2 on mitochondrial respiration and thus suggest that the inhibition of complex I activity is

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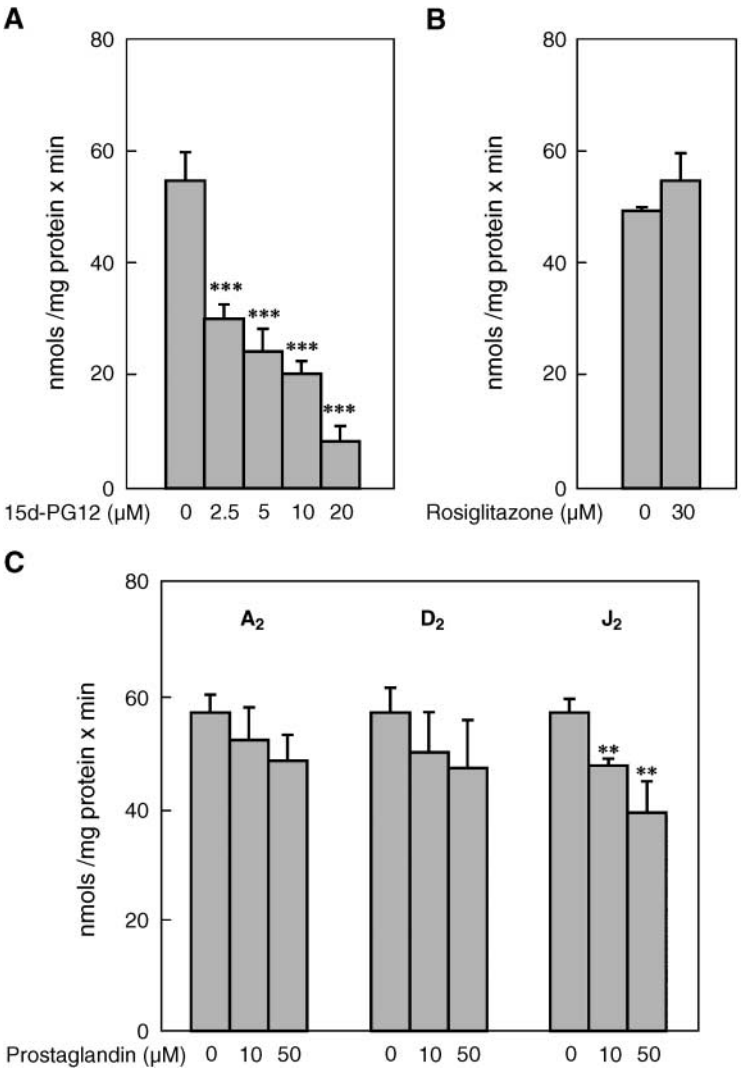

Fig. 2. Effect of 15d-PGJ_2 on complex I activity. The enzymatic activity of complex I was determined in nonsynaptic mitochondria isolated from cerebral cortex as indicated in Experimental Procedures. The *y* axes represent nanomoles of NADH oxidized per milligram of protein per minute. A: Effect of different concentrations of 15d-PGJ_2 on complex I activity. B: Effect of rosiglitazone on complex I activity. C: Effect of prostaglandins A_2, D_2 and J_2 on complex I activity. The values shown represent means of at least five different experiments in duplicate, and the bars represent standard errors of the mean. ** $P \leq 0.01$ and *** $P \leq 0.001$ versus activity in the absence of prostaglandin.

a major target for 15d-PGJ_2 action in the mitochondria. We next analyzed the effect on complex I activity of other prostaglandins. As shown in Fig. 2C, neither prostaglandin A_2 nor prostaglandin D_2 showed a significant inhibitory effect on complex I activity at concentrations as high as 50 μM. PGJ_2 showed a modest inhibitory effect, and a concentration of 50 μM was required to obtain an ~30% inhibition of complex I activity.

The inhibitory effect of 15d-PGJ_2 on complex I activity was analyzed at different concentrations of the electron acceptor decylubiquinone. As shown in **Fig. 3**, when the data were represented as 1/complex I activity (1/V) versus 1/[decylubiquinone] (1/[Q]), parallel lines were obtained in the absence and presence of two concentrations of 15d-PGJ_2, suggesting a noncompetitive mechanism for the inhibitory action of this prostaglandin.

15d-PGJ_2 has two reactive α,β-unsaturated carbonyl moieties that can act as Michael acceptors by reacting with thiol groups (33, 34). 15d-PGJ_2 has been reported to use this mechanism to modify the function of diverse proteins (17, 21). This reaction is sensitive to the reducing agent DTT, which has been shown to eliminate the inhibitory effect of this prostaglandin on NF-κB binding to DNA (17). Therefore, we next analyzed the possible effect of DTT on the inhibitory effect of 15d-PGJ_2 on complex I activity. As shown in **Fig. 4**, when mitochondrial membranes preincubated with 15d-PGJ_2 were washed in the presence of 1 mM DTT, no inhibitory effect of 15d-PGJ_2 was observed. In contrast, the inhibitory effect remained when the membranes were washed with the same buffer but in the absence of DTT, suggesting the formation by 15d-PGJ_2 of an adduct with a critical component of complex I as a probable mechanism to account for the action of this prostaglandin.

ROS production

As previously indicated, 15d-PGJ_2 increases ROS production (22–27); therefore, we next tested whether complex I, a major ROS producer in the cell, could be implicated in this action. Consequently, we determined the effect of 15d-PGJ_2 on mitochondrial ROS production in freshly prepared nonsynaptic mitochondria isolated from rat cerebral cortex. As shown in **Fig. 5**, 15d-PGJ_2 increases the production of ROS by 8-fold, as measured by the rate of hydrogen peroxide production in the presence of glutamate/malate and ADP. As expected, the classical com-

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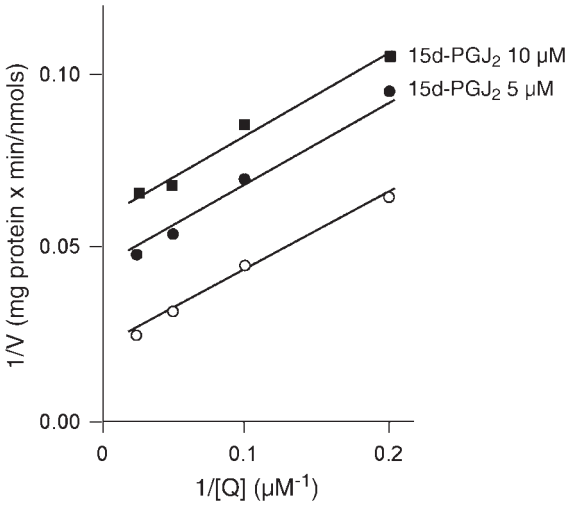

Fig. 3. Effect of different concentrations of decylubiquinone on the inhibitory effect of 15d-PGJ_2 on complex I activity. The enzymatic activity of complex I was determined in nonsynaptic mitochondria isolated from cerebral cortex as indicated in Experimental Procedures. The *y* axis represents the inverse of complex 1 activity (nanomoles of NADH oxidized/milligram of protein per minute) and the x-axis is 1/[decylubiquinone]. Open circles represent the activity in the absence of 15d-PGJ_2.

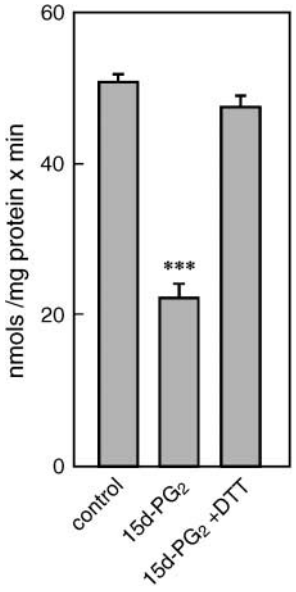

Fig. 4. Effect of dithiothreitol on 15d-PGJ_2 inhibition of complex I activity. Mitochondrial membranes (50 μg of protein) were incubated with or without 10 μM 15d-PGJ_2 at 22°C for 1 min in a final volume of 100 μl and immediately washed either twice with 1 ml of the assay buffer without NADH or decylubiquinone or once with 1 ml of the same buffer plus 1 mM DTT and once with 1 ml of the buffer without DTT. Complex I activity was then determined as indicated in Experimental Procedures. The *y* axis represents nanomoles of NADH oxidized per milligram of protein per minute. The values shown represent means of at least three different experiments in duplicate, and the bars represent standard errors of the mean. *** $P \leq 0.001$ versus control.

plex I inhibitor rotenone (10 μM) also induced the production of ROS (Fig. 5B), although with a lower efficiency (3-fold in contrast with the 8-fold induction by 15d-PGJ_2). The same results were obtained with 2 μM rotenone (data not shown). When both compounds, rotenone and 15d-PGJ_2, were simultaneously added to the reaction, only a 4.7-fold increase was observed, indicating that rotenone inhibited ROS production induced by 15d-PGJ_2. These results suggest that 15d-PGJ_2 induces mitochondrial ROS production mainly at the level of complex I.

In agreement with the results obtained analyzing complex I activity, PGA_2 and prostaglandin D_2 had no effect on mitochondrial ROS production. PGJ_2 also had an effect on ROS production, although to a lesser extent (Fig. 5C).

DISCUSSION

In this work, we have shown that 15d-PGJ_2 is a potent inhibitor of mitochondrial complex I activity and a strong inducer of ROS production, probably at the level of this complex.

When the complex I substrates glutamate/malate and pyruvate were assayed, 15d-PGJ_2 blocked state 3 mitochondrial respiration. In contrast, when succinate, a complex II substrate, was added in the presence of rotenone, a complex I inhibitor, 15d-PGJ_2 did not inhibit state 3 respiration. No effect of 15d-PGJ_2 was observed on oxygen consumption in respiratory state 4, suggesting that this compound does not have an uncoupling effect. In contrast, other lipids known to inhibit the respiratory chain also altered the permeability of the mitochondrial membrane, causing an uncoupling effect (35, 36).

In addition to inhibiting complex I activity, 15d-PGJ_2 strongly induces mitochondrial ROS production. Complex I is considered one of the major places of ROS production in the mitochondrion and, therefore, one of the major producers of ROS within the cell (37–39). Here, we show that the classical complex I inhibitor rotenone clearly reduced ROS production induced by 15d-PGJ_2 in isolated mitochondria, in agreement with recent work from our laboratory showing that rotenone in MCF-7 cells is a potent inhibitor of 15d-PGJ_2-induced oxidative stress (40). These results clearly implicate complex I as the site of ROS production induced by 15d-PGJ_2. Our results are in agreement with previous results by other groups showing that 15d-PGJ_2, at micromolar concentrations, induces an increase in cellular ROS production (22–26) and particularly with the suggestion by Kondo et al. (22) that the mitochondrion is the place of ROS production induced by this prostaglandin.

Regarding the biological consequences of the effects described here, we propose that these mitochondrial effects could be implicated in the loss of cell viability and the induction of antioxidant enzymes by 15d-PGJ_2 shown in diverse cell lines (14, 41–44). In this regard, it is interesting that the addition of antioxidant compounds, such as *N*-acetylcysteine, *N*-(2-mercapto-propionyl)-glycine, and pyrrolidine dithiocarbamate, prevent the induction by 15d-PGJ_2 of cell death in human neuroblastoma SH-SY5Y and

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human hepatic myofibroblasts (22, 23), the activation of mitogen-activated protein and stress-activated kinases (25), and the decrease in mitochondrial potential in Jurkat T-cells (27). In addition to these data, we recently showed that rotenone and the radical scavengers ebselen and ascorbate prevented the induction of cell death in MCF-7 cells (40), a finding that clearly supports the proposal that the mitochondrial effects of this prostaglandin are important mediators in the loss of cell viability.

The possible mechanism of 15d-PGJ_2 action on complex I activity was analyzed by studying the action of other PPARγ ligands on isolated mitochondria and the sensitivity of 15d-PGJ_2 action to the reducing agent DTT. The presence of PPARγ receptors within the mitochondrial matrix of specific tissues has been described, although their possible physiological function is unclear (32). Here, we show that the high-affinity PPARγ ligand rosiglitazone, at a concentration known to efficiently induce gene expression (12), had no effect on oxygen consumption and the activity of complex I, suggesting that the inhibition of complex I by 15d-PGJ_2 is PPARγ-independent. The present data also indicate that the observed effects of 15d-PGJ_2 on mitochondrial complex I activity is specific for this prostaglandin, because other prostaglandins, such prostaglandins A_2 and D_2, do not exhibit this effect and the structurally more related PGJ_2 showed only a modest effect.

15d-PGJ_2 has electrophilic carbon moieties, which are susceptible to undergoing addition reactions (Michael addition) with nucleophiles such as the free thiol groups (33, 34). In this way, 15d-PGJ_2 has been shown to modify the function of diverse proteins (17, 21). This reaction is sensitive to the reducing agent DTT, which has been shown to eliminate the inhibitory effect of this prostaglandin on NF-κB binding to DNA (17). Therefore, the results presented here show that the inhibitory effect of this prostaglandin on complex I activity is washed out in the presence of 1 mM DTT, suggesting the formation by 15d-PGJ_2 of an adduct with a critical component of mitochondrial complex I.

In summary, our data support the hypothesis that 15d-PGJ_2 could form a covalent complex with the respiratory complex I and, as a consequence, inhibit the function of this complex and increase ROS production, an effect that probably represents an important mechanism for the observed cytostatic effects of this prostaglandin.

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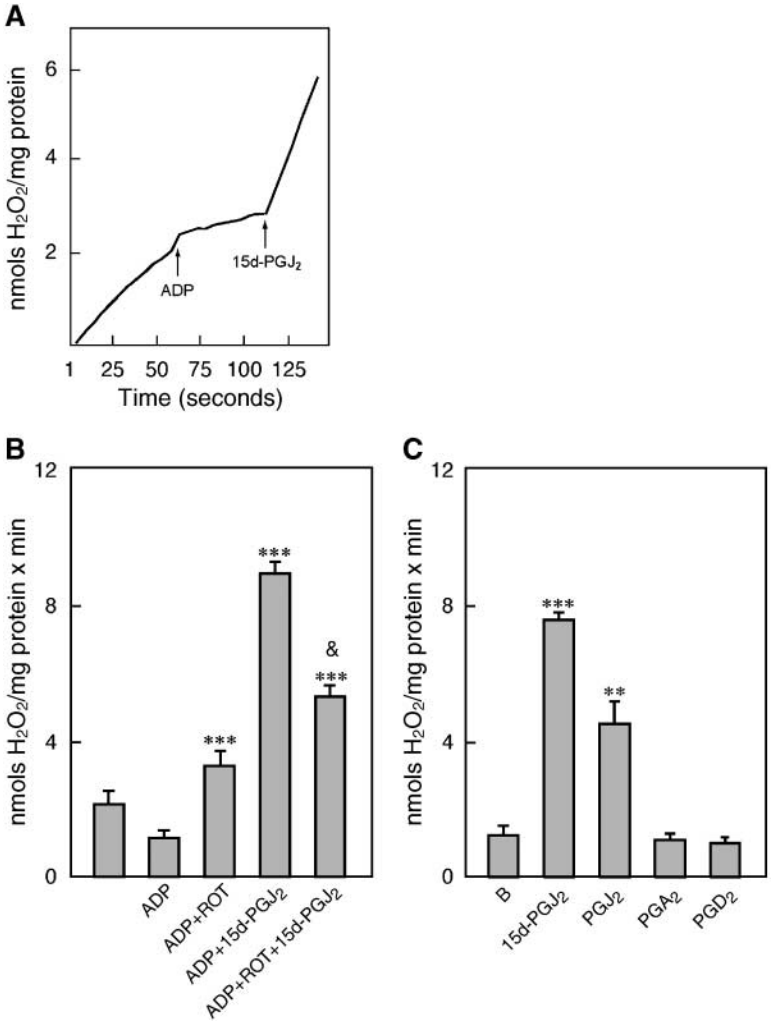

Fig. 5. Effect of 15d-PGJ_2 on hydrogen peroxide production. Nonsynaptic mitochondria were isolated from rat cerebral cortex, and the rate of hydrogen peroxide production was determined as indicated in Experimental Procedures. A: Kinetics of reactive oxygen species (ROS) production in the presence of glutamate/malate (5 mM each) and after the sequential addition of ADP (0.5 mM) and 15d-PGJ_2 (10 μM). B: ROS production rate in the presence of glutamate/malate and after the addition of different combinations of ADP, 15d-PGJ_2, and rotenone (ROT; 10 μM). C: ROS production rate in the presence of glutamate/malate and ADP and after the addition of the indicated prostaglandin at a concentration of 10 μM. The values shown represent means of at least three different experiments in duplicate, and the bars represent standard errors of the mean. ** $P \leq 0.01$ and *** $P \leq 0.001$ versus ROS production rate in the presence of ADP; $^{\&}$ $P \leq 0.01$ versus ROS production rate in the presence of ADP plus 15d-PGJ_2; B, basal.

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