Modulation of nitric oxide concentration and lipid metabolism by 15-deoxy \( \Delta^{12,14} \)prostaglandin \( J_2 \) in embryos from control and diabetic rats during early organogenesis

A. Jawerbaum, D. Sinner, V. White, C. Pustovrh, E. Capobianco and E. Gonzalez

Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Serrano 669, (1414) Buenos Aires, Argentina

The concentration of 15-deoxy \( \Delta^{12,14} \)PGJ\(_2\) (15dPGJ\(_2\)) and its effects on nitric oxide generation and neutral lipid in embryos from control and neonatal streptozotocin-induced (n-stz) diabetic rats during organogenesis were investigated. 15dPGJ\(_2\) is produced in embryos during organogenesis, and its production is lower in embryos of n-stz diabetic rats than in embryos from control rats. Nitrate and nitrite concentrations were higher in embryos from n-stz diabetic rats and were reduced in the presence of 15dPGJ\(_2\) both in embryos from control and diabetic rats. Thus, decreased 15dPGJ\(_2\) concentrations in embryos from n-stz diabetic rats may be related to the high nitric oxide concentrations found in those embryos. Exogenous 15dPGJ\(_2\) decreased cholesterol and cholesteryl ester concentrations in embryos from control and n-stz diabetic rats, and reduced triacylglycerol concentrations in control embryos. Incorporation of [\(^{14}\)C]acetate into lipids showed decreased \textit{de novo} synthesis of cholesteryl ester and triacylglycerides in embryos from n-stz diabetic rats compared with controls. Exogenous 15dPGJ\(_2\) reduced the incorporation of [\(^{14}\)C]acetate into triacylglycerides, cholesterol and cholesteryl ester in embryos from both control and n-stz diabetic rats. 15dPGJ\(_2\) is present in embryos during organogenesis, and reduces embryonic nitric oxide production and lipid synthesis. The lower 15dPGJ\(_2\) concentration in embryos from n-stz diabetic rats may result in developmental alterations in this diabetic model.

Introduction

The cyclopentenone, 15-deoxy \( \Delta^{12,14} \)prostaglandin \( J_2 \) (15dPGJ\(_2\)), is a metabolite of prostaglandin \( D_2 \) (PGD\(_2\)). In contrast to classical prostaglandins, which bind to cell surface \( \mathrm{G} \) protein-coupled receptors, 15dPGJ\(_2\) is a natural ligand of a nuclear receptor, the peroxisome-proliferator activated receptor gamma (PPAR\( \gamma \)) (Forman et al., 1995; Kliewer et al., 1995). PPAR\( \gamma \) behaves as a ligand-activated transcription factor through its DNA binding domain, which recognizes response elements in the promoter of specific target genes linked to apoptosis, cell proliferation, differentiation, inflammation, lipid metabolism and glucose homeostasis (Kliewer and Wilson, 1998). PPAR\( \gamma \) was identified initially in differentiated adipocytes, and its role has been determined in relation to the pathogenesis of insulin resistance because members of the thiazolidinedione class of antidiabetic drugs such as troglitazone have been identified as a ligand of PPAR\( \gamma \) (Lehmann et al., 1995).

In addition to hyperglycaemia, type 2 diabetic individuals almost invariably manifest a serious breakdown in lipid metabolism, often reflected by increased concentrations of circulating free fatty acids and triacylglycerides, together with excessive fat deposition in various tissues including muscle (McGarry, 2002). Such alterations are ameliorated by thiazolidinedione treatment (Mimura et al., 1994; Cha et al., 2001). Lipids play an essential role in embryonic growth and development as components of the newly formed cell membranes, oxidative fuels that support embryo growth and possibly as signalling molecules (Herz and Farese, 1999). Indeed, defects in endogenous cellular biosynthesis, receptor-mediated endocytosis and receptor-mediated transfer of neutral lipids, either through the effect of pharmacological agents or through the analysis of induced or naturally occurring genetic mutations, lead to disrupted development (Barbu et al., 1984; Irons et al., 1993; Farese et al., 1996).

There is an increased incidence of congenital malformations in the offspring of diabetic patients and in experimental models of diabetes (Kitzmiller et al., 1978; Eriksson et al., 1991). Although it is clear that alterations in the synthesis of the lipid components of the pulmonary complexes in fetuses from diabetic mothers at term have been related to an increased incidence of respiratory
distress syndrome (Singh and Feigelson, 1983), little is known about the modulatory factors that influence lipid metabolism during early organogenesis, the period when most malformations are induced.

Administration of streptozotocin to 2-day-old neonates, a non-insulin-dependent diabetic model, results in reproductive abnormalities. Adult diabetic rats are mildly hyperglycaemic, mildly hypoinsulinaemic and glucose intolerant (Portha et al., 1979). During pregnancy, uterine contractile activity and lipid metabolism are impaired, and there is an abnormal production of prostanoid in uterine, placental and amniotic tissues (Jawerbaum et al., 1994). There are also increased resorption rates and enhanced PGE$_2$ production in embryos obtained from n-stz diabetic rats during organogenesis (Jawerbaum et al., 1998). Indeed, these high concentrations of PGE$_2$ appear to be the result of an overproduction of nitric oxide (NO) that positively modulates PGE$_2$ generation (Jawerbaum et al., 1998). The nuclear factor kappaB (NF-kB) and its activating kinase are key targets for the anti-inflammatory activity of 15dPGJ$_2$, which inhibits NF-kB-mediated transcriptional activation by PPARgamma-dependent and -independent molecular mechanisms (Ricote et al., 1998; Rossi et al., 2000). There are reduced concentrations of 15dPGJ$_2$ in B cells isolated from diabetic rats, and addition of 15PGJ$_2$ inhibits NO and PGE$_2$ production, ameliorating the inflammatory response that leads to B-cell destruction (Gonzalez et al., 2001). In the present study, 15dPGJ$_2$ concentration and the effect of exogenous 15dPGJ$_2$ on NO concentration, the lipid profile and the synthesis of labelled lipids from [1,2-$^{14}$C]acetate in embryos obtained from control and neonatal streptozotocin-induced (n-stz) diabetic rats during early organogenesis were evaluated as part of an attempt to identify an endogenous inducer of NO overproduction and anomalies of lipid metabolism in embryos from diabetic rats.

### Materials and Methods

**Materials**

Streptozotocin and citrate buffer were obtained from Sigma (St Louis, MO). Glucostix reagent strips were obtained from Bayer Diagnostics (Boston, MA). 15-Deoxy-D$_{12,14}$prostaglandin J$_3$ Enzyme Immunoassay Kit was purchased from Assay Designs Inc. (Ann Arbor, MI). Nitrate-nitrite assay kit was purchased from Cayman Chemical Co. (Ann Arbor, MI). Phosphatidyl choline, phosphatidyl ethanolamine, sphingomyelin, phosphatidyl inositol, triacylglycerol, fatty acids, cholesteryl ester and cholesterol standards were purchased from Doosan Serdary Research Laboratories (Yongin). Thin-layer chromatography (TLC) plates Silica gel 60 were obtained from Merck (Darmstadt).

**Animals**

Albino Wistar rats bred in the laboratory were fed Purina rat chow ad libitum. At 2 days of age the neonates from rats bred in the laboratory were injected i.p. with either 100 mg streptozotocin kg$^{-1}$ in 0.05 mol citrate buffer l$^{-1}$, pH 4.5 or with buffer alone (controls). Four days after birth, neonates exhibiting glycosuria (> 500 mg glucose dl$^{-1}$) (Diastix reagent strips; Bayer Diagnostics) were considered diabetic. Spontaneous response to this treatment led to a non-insulin-dependent diabetic state in the adult, which was stable and chronic, as described by Portha et al. (1979). The adult diabetic state was confirmed by means of glucose tolerance tests (Maloff and Boyd, 1986). Glycaemia was determined in blood from tail veins by Glucostix reagent strips (Bayer Diagnostics) and a glucometer. Basal blood glucose concentrations of diabetic rats were 215 ± 21 mg dl$^{-1}$, whereas blood glucose concentrations of control rats were 100 ± 10 mg dl$^{-1}$. Two hours after a glucose challenge (1.5 g glucose kg$^{-1}$ i.p.) blood glucose concentrations of diabetic rats were > 250 mg dl$^{-1}$, whereas blood glucose concentrations of control rats were < 110 mg dl$^{-1}$. Control rats were injected with buffer alone. Control and n-stz diabetic rats were caged overnight with normal male rats. Mating was confirmed by the presence of spermatozoa in vaginal smears. When a positive pregnancy was identified, this was considered day 0.5 of gestation. The guidelines for the care and use of animals approved by the local institution were followed, according to Principles of Laboratory Animal Care (NIH publication number 85–23, revised 1985).

**Embryo preparations**

Animals were killed by cervical dislocation on day 10.5 of pregnancy and the uterus of each animal was transferred to a Petri dish containing Krebs–Ringer bicarbonate (KRB) solution: 11.0 mmol glucose l$^{-1}$, 145 mmol Na$^+$ l$^{-1}$, 2.2 mmol Ca$^{2+}$ l$^{-1}$, 1.2 mmol Mg$^{2+}$ l$^{-1}$, 127 mmol Cl$^-$ l$^{-1}$, 25 mmol HCO$_3^-$ l$^{-1}$, 1.2 mmol SO$_4^{2-}$ l$^{-1}$ and 1.2 mmol PO$_4^{3-}$ l$^{-1}$. A stereomicroscope and watchmaker forceps were used to remove the balls of decidual tissue from each uterus and to open them carefully to free the conceptuses. The embryos were dissected out of the yolk sacs and evaluated morphologically under a stereomicroscope. Viability was established by the presence of a beating heart. The embryos were categorized as morphologically normal or as showing neural tube defects or other malformations. Embryonic growth was quantified by direct measurement of
the protein content (Bradford, 1976), and bovine serum albumin was used as a standard. Embryos in resorption stages were denoted as resorbed and received no further analysis. The viable embryos were stored at −70°C to determine 15dPGJ2 concentration, or incubated as described below.

Four embryos obtained from control or diabetic rats were incubated in a metabolic shaker, under an atmosphere of 5% CO2 in 95% O2 at 37°C for 3 h, in 0.5 ml KRB with or without the addition of 2 × 10−6 mol 15dPGJ2 l−1. After incubation, the embryos were stored at −70°C until determination of nitrate–nitrite concentration or lipid extraction.

The de novo synthesis of labelled lipids from [14C]acetate was evaluated by incubating four embryos for 3 h in the same conditions as described above, with or without the addition of 2 × 10−6 mol 15dPGJ2 l−1 and 1.2 μCi [14C]acetate ml−1 (55.2 mCi mmol−1). After incubation, the embryos were washed three times in 10 ml KRB to remove adherent [14C]acetate and then stored at −70°C until lipid extraction.

### 15-deoxy Δ12,14 prostaglandin J2 determinations

15dPGJ2 was measured in the control and n-stz diabetic embryos using the 15-deoxy Δ12,14 prostaglandin J2 Enzyme Immunoassay Kit (Assay Designs Inc.) Five embryos were sonicated in PBS; an aliquot was separated for protein determination as described by Bradford (1976); and embryonic prostaglandins were extracted twice in absolute ethanol. The extracts were dried in a Savant (Hicksville, NY) Speed-Vac concentrator and stored at −70°C until enzyme-immunoassay. When the assay was performed, the extracts were reconstituted with 50 μl ethanol and 200 μl assay buffer provided by the commercial kit. In brief, the kit provides a polyclonal antibody to 15dPGJ2 to bind in a competitive manner the prostaglandin in the sample or an alkaline phosphatase molecule to which 15dPGJ2 is covalently attached. After a simultaneous incubation, a p-nitrophenyl phosphate substrate is added, and the yellow colour generated is evaluated on a microplate reader at 405 nm. The results in the present study were expressed as pg per μg protein. Crossreactivities reported by Assay Designs are: 100% for 15dPGJ2, 49.2% for PGJ2, 5.99% for Δ12,14PGJ2 and 4.92% for PGD2.

### Nitrate–nitrite assay

The embryonic nitrate and nitrite concentrations were quantified using an assay kit for nitrate and nitrite determinations (Cayman Chemical). The embryos were sonicated in Tris–HCl buffer solution, and an aliquot was separated for protein determination. Nitrates in the supernatant were reduced to nitrites using nitrate reductase, and total nitrites were measured by the Griess method (Green et al., 1982). Absorbance was measured at 540 nm in a microtitre plate using NaNO3 and NaNO2 as standards. Results were expressed in nmol per μg protein.

### Lipid analysis

Total lipids were extracted in methanol–chloroform 2:1 (v/v) (Blight and Dyer, 1959) and then concentrated in a Savant Speed-Vac concentrator. Neutral and total polar lipids were quantified by TLC and fluorescence emission of 1,6-diphenylhexatriene (DPH) as described by Igal et al. (2001). In brief, chromatography of the total embryo lipids was performed using a solvent system consisting of hexane–ethyl ether–acetic acid–water 50:37.5:3.5:1.5 (v/v/v/v). Chromatography was resolved on TLC plates with chloroform–methanol–acetic acid–water 50:37.5:3.5:1.5 (v/v/v/v). Lipid species were quantified by comparison with known amounts of pure lipid standards run on the same plate. As a control for the fluorescence method, the lipids were also stained with iodine and with a ferric chloride solution and the spot images were processed in the same way as for the fluorescent spots; < 15% essential differences between the methods were apparent.

Neutral lipids were separated for radioactivity measurements by TLC as described above. Polar lipid species were resolved on TLC plates with chloroform–methanol–acetic acid–water 50:37.5:3.5:1.5 (v/v/v/v). Chromatography was carried out in all samples in parallel with pure lipid standards. [14C]-labelled lipids were detected with a

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### Table 1. 15-deoxy Δ12,14 PGJ2 concentrations and morphological characteristics of embryos from control and n-stz diabetic rats on day 10.5 of gestation

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Embryos from control rats</th>
<th>Embryos from diabetic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resorptions (embryos)</td>
<td>1% (1/127)</td>
<td>10%b (17/165)</td>
</tr>
<tr>
<td>Malformations (embryos)</td>
<td>1% (1/126)</td>
<td>10%b (15/148)</td>
</tr>
<tr>
<td>Number of somites (embryos)</td>
<td>14 ± 1 (64)</td>
<td>12 ± 0.5 (73)</td>
</tr>
<tr>
<td>Protein content (μg) (embryos)</td>
<td>66 ± 4 (21)</td>
<td>50 ± 2a (21)</td>
</tr>
<tr>
<td>15-deoxy Δ12,14 PGJ2 (pg per μg embryonic protein)</td>
<td>22.6 ± 3.6 (8)</td>
<td>9.1 ± 2a (8)</td>
</tr>
</tbody>
</table>

Values are percentage of embryos (chi-squared statistics) or means ± SEM. (Student’s t test) for embryos obtained randomly from 8–10 rats: aP < 0.01; bP < 0.005 versus embryos from control rats.
Berthold (Bad Wildbad) Scanner II. Radioactive spots were scraped into vials and counted in a liquid scintillation counter.

Statistical analyses

All values presented in this study are mean ± SEM. Comparisons among groups were performed using one-way ANOVA in conjunction with the Tukey’s test. Differences between groups were considered significant when P < 0.05.

Results

15-deoxy Δ12,14-prostaglandin J2 concentrations and morphological characteristics of embryos from control and n-stz diabetic rats

Embryos obtained from n-stz diabetic rats on day 10.5 of gestation showed a higher rate of resorption (P < 0.01), a higher rate of embryonic malformation (P < 0.01), reduced protein content (indicating growth delay, P < 0.01), and no alteration in the number of somites when compared with embryos from control rats (Table 1).

15dPGJ2 concentrations were lower in the embryos from n-stz diabetic rats compared with controls (P < 0.01) (Table 1).

Effect of 15-deoxy Δ12,14-prostaglandin J2 on nitrate–nitrite concentrations in embryos from control and n-stz diabetic rats

Embryos obtained from control and n-stz diabetic rats on day 10.5 of gestation were incubated for 3 h in Krebs-Ringer bicarbonate solution with or without 2 × 10^{-6} mol 15-deoxy Δ12,14-prostaglandin J2 (15dPGJ2) l^{-1}. Values are mean ± SEM for 36–40 embryos obtained randomly from 9–10 rats. Asterisks indicate significant difference from embryos from control rats without addition of 15dPGJ2: *P < 0.05, **P < 0.02; daggers indicate significant difference from embryos from diabetic rats without addition of 15dPGJ2: †††P < 0.001 (ANOVA).

Effect of 15-deoxy Δ12,14-prostaglandin J2 on the incorporation of [14C]acetate into lipids in embryos from control and diabetic rats during organogenesis

Embryos obtained from control and n-stz diabetic rats on day 10.5 of gestation were incubated for 3 h in Krebs’ medium added with tracer amounts of [14C]acetate to evaluate de novo lipid synthesis in the presence or absence of 2 × 10^{-6} mol 15dPGJ2 l^{-1}. The rate of incorporation of [14C]acetate to triacylglycerol (P < 0.01) and cholesteryl ester (P < 0.05) was lower in stz-diabetic embryos than in controls, whereas no alterations in [14C]acetate incorporation to cholesterol were detected (Fig. 3).

The addition of 2 × 10^{-6} mol 15dPGJ2 l^{-1} induced a decrease in [14C]acetate incorporation to triacylglycerol (64%, P < 0.001), cholesterol (55%, P < 0.01) and cholesteryl ester (68%, P < 0.001) in embryos from control rats (Fig. 3). Similarly, in embryos from n-stz diabetic rats, exogenous 2 × 10^{-6} mol 15dPGJ2 l^{-1} reduced the incorporation of [14C]acetate to triacylglycerol (57%, P < 0.01), cholesterol (67%, P < 0.01) and cholesteryl ester (60%, P < 0.01) compared with diabetic embryos incubated without additions (Fig. 3).
In the present study, rat embryos were shown to produce 15dPGJ2 during organogenesis. Embryonic 15dPGJ2 production was similar to that of other prostaglandins such as PGE2 (Jawerbaum et al., 1998). 15dPGJ2, like other cyclopentenones, lacks cell surface receptors but is actively transported into cells, where it exerts a wide variety of biological actions, including modulation of lipid and glucose homeostasis, cell differentiation, cessation of cell growth, antitumoral activity and anti-inflammatory activity (Straus and Glass, 2001). However, the role of 15dPGJ2 during embryonic development is not known. 15dPGJ2 is sequentially formed from PGD2, PGJ2 and D12PGJ2 in vivo, and has been identified in inflammatory fluids in increasing concentrations during the resolution phase of inflammation (Shibata et al., 2002). Its precursor, D12PGJ2, which is increased by in vivo administration of PGD2, is a natural component of human body fluids, and its generation is suppressed by treatment with cyclooxygenase (COX) inhibitors (Hirata et al., 1988). In the n-stz diabetic rat model evaluated in the present study, in addition to morphological abnormalities and growth delay, embryonic 15dPGJ2 concentrations were decreased. 15dPGJ2 concentrations are lower in β cells obtained from diabetic rats (Gonzalez et al., 2001) and exogenous 15dPGJ2 reduces PGE2 and NO concentrations, an effect that involves inhibition of iNOS and COX-2 (Gonzalez et al., 2001). Thus, a decreased concentration of 15dPGJ2 in diabetic β cells appears to be related to the increase in NO and PGE2 concentrations, resulting in cellular damage and β-cell death.

Discussion

In the present study, rat embryos were shown to produce 15dPGJ2 during organogenesis. Embryonic 15dPGJ2 production was similar to that of other prostaglandins such as PGE2 (Jawerbaum et al., 1998). 15dPGJ2, like other cyclopentenones, lacks cell surface receptors but is actively transported into cells, where it exerts a wide variety of biological actions, including modulation of lipid and glucose homeostasis, cell differentiation, cessation of cell growth, antitumoral activity and anti-inflammatory activity (Straus and Glass, 2001). However, the role of 15dPGJ2 during embryonic development is not known. 15dPGJ2 is sequentially formed from PGD2, PGJ2 and D12PGJ2 in vivo, and has been identified in inflammatory fluids in increasing concentrations during the resolution phase of inflammation (Shibata et al., 2002). Its precursor, D12PGJ2, which is increased by in vivo administration of PGD2, is a natural component of human body fluids, and its generation is suppressed by treatment with cyclooxygenase (COX) inhibitors (Hirata et al., 1988). In the n-stz diabetic rat model evaluated in the present study, in addition to morphological abnormalities and growth delay, embryonic 15dPGJ2 concentrations were decreased. 15dPGJ2 concentrations are lower in β cells obtained from diabetic rats (Gonzalez et al., 2001) and
NO concentration is increased in the embryos from both insulin and non-insulin-dependent experimental models of diabetes, an alteration probably related to the increase in apoptosis, resorption and malformation rates induced by diabetes (Jawerbaum et al., 1998, 2001; Moley, 2001). The potent anti-inflammatory activity of 15dPGJ2 in different types of cell prompted the present authors to evaluate the relationship between 15dPGJ2 and NO concentrations in normal and diabetic embryos during organogenesis. 15dPGJ2 was found to inhibit NO generation in embryos from both control and n-stz diabetic rats. The lower 15dPGJ2 concentration in embryos from n-stz diabetic rats is likely to be related to their increased NO concentration and may affect embryo development in diabetic mothers.

15dPGJ2 and thiazolidinediones are pharmacological activators of PPARγ and modulate lipid and glucose metabolism, and promote adipocyte differentiation (Forman et al., 1995; Lehmann et al., 1995). PPARα plays a major role in fatty acid catabolism and PPARγ plays a major role in adipogenesis and lipid storage (Wahli et al., 1995). However, thiazolidinediones induce enhancement of fatty acid oxidation in non-adipose tissues such as skeletal muscle (Cha et al., 2001).

During development, lipids serve primarily as modifiers of the fluidity, structure and function of membranes. Although alterations in lipid metabolism affect development at term of the fetus of a diabetic mother (Singh and Feigelson, 1983), little is known regarding lipid metabolism in embryos from diabetic mothers during organogenesis. In the present study, no alterations in the content of triacylglycerol, cholesterol and cholesteryl esters in the embryos obtained from n-stz diabetic rats compared with controls were observed, but there was a reduced rate of incorporation of [14C]acetate to triacylglycerol and cholesteryl ester in the embryos from n-stz diabetic rats. A decreased embryonic lipid synthesis may be a metabolic response to increased maternal–embryo lipid transfer and may help to prevent an increase in the embryonic lipid concentrations. Indeed, triglyceride accumulation is present in pregnant uterine tissues and in the fetoplacental unit in n-stz diabetic animals (Jawerbaum et al., 1994), and this is not reflected in the embryonic lipid profile. In contrast, in a more severe model of diabetes, there is a significant positive correlation between maternal plasma circulating lipids and fetal tissue lipid content (Shafrir and Barash, 1978).

In the present study, 15dPGJ2 was demonstrated to decrease neutral lipid content in control and n-stz diabetic embryos during organogenesis. This decrease occurs via a mechanism that involves an inhibition of neutral lipid synthesis, as de novo incorporation of [14C]acetate to embryonic triacylglycerides, cholesterol and cholesteryl ester was decreased. Thiazolidinediones reduce hypertriglyceridaemia (Oakes et al., 2001) via mechanisms that involve an enhanced ability of adipose tissue to take up and store triacylglycerides in newly differentiated adipocytes, and a reduction of triacylglyceride content in peripheral tissues such as pancreatic islets (Shimabukuro et al., 1997) and liver (Oakes et al., 2001). In addition, troglitazone inhibits cholesterol biosynthesis in cultured Chinese hamster ovary cells (Wang et al., 1999).

The inhibitory effect of 15dPGJ2 on embryonic cholesterol content and synthesis is particularly interesting. Cholesterol is needed in sonic hedgehog (SHH) signalling pathways, and mutations in the SHH gene exhibit an indistinguishable phenotype from that seen in Smith–Lemli–Opitz syndrome (Farese and Herz, 1998), an autosomal recessive multiple congenital malformation syndrome associated with reduced cholesterol biosynthesis (Irons et al., 1993). Indeed, inhibitors of delta 7-dehydrocholesterol reductase, an enzyme involved in the last step of the cholesterol biosynthesis pathway, are inducers of fetal malformations (Barbu et al., 1984).

In the present study, reduced concentrations of 15dPGJ2 were demonstrated in embryos from n-stz diabetic rats. Although much work is needed to understand the biological significance of this finding completely, the low concentration of 15dPGJ2 may be related to NO overproduction that alters embryo development. In addition, the profound effects of 15dPGJ2 on embryonic lipid content and synthesis during organogenesis indicated a significant role for 15dPGJ2 in this important pathway providing developmental signals, energy storage and structural components for normal embryonic development.

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