COMMENTARY

The Dilemma of Oxidative Stress Personified by the Diprosopus 8-*iso*-Prostaglandin $F_{2\alpha}$ and Prostaglandin $F_{2\alpha}$

Dimitrios Tsikas

Core Unit Proteomics, Hannover Medical School, Hannover, Germany

Abstract

In general, the term "oxidative stress" describes an imbalance between oxidants and antioxidants in favor of the oxidants. While antioxidant defense is widely accepted to involve both enzymatic and non-enzymatic reactions, oxidants are generally assumed to be produced by non-enzymatic processes involving chemically produced free radicals. However, many oxidants are also formed by numerous enzymes and proteins. The F_2-isoprostane 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) and malondialdehyde (MDA) are widely used as biomarkers of oxidative stress, although there is evidence that both 8-*iso*-$PGF_{2\alpha}$ and MDA are also produced enzymatically from arachidonic acid by the action of cyclooxygenase (COX). On the contrary, there is also evidence that $PGF_{2\alpha}$ is produced from arachidonic acid both by the action of COX and non-enzymatically. The duality of oxidative stress, personified by 8-*iso*-$PGF_{2\alpha}$ and $PGF_{2\alpha}$, is a serious dilemma and demands new definitions and strategies from the scientists.

Keywords: 8-iso-prostaglandin F2α; arachidonic acid; cyclooxygenase inhibitors; oxidative stress; prostaglandin F2α

Introduction

There is solid evidence that 8-*iso*-prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is produced both enzymatically and non-enzymatically *in vivo* in animals and in humans. This evidence is based on the inhibitory action of cyclooxygenase (COX) inhibitors and has been reported by many groups (1–3). For many decades, $PGF_{2\alpha}$ has been thought to be exclusively formed from arachidonic acid by the catalytic action of COX. Yet, there is also solid evidence that $PGF_{2\alpha}$ is an abundant non-enzymatic oxidation product of arachidonic acid (4). van't Erve et al. (5) proposed that the 8-*iso*-$PGF_{2\alpha}$/$PGF_{2\alpha}$ molar ratio can be used to distinguish chemical from enzymatic lipid peroxidation. Yet, the key information that the isomeric prostaglandins 8-*iso*-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ can be produced from arachidonic acid via enzymatic and non-enzymatic processes has not been considered by van't Erve and colleagues (5). It is worth mentioning that these authors have demonstrated that recombinant prostaglandin H synthase (PGHS) isoforms 1 (PGHS-1) and 2 (PGHS-2), also known as COX-1 and COX-2, respectively, catalyze the formation of 8-*iso*-$PGF_{2\alpha}$ from free arachidonic acid (5). This finding confirms our recent finding on recombinant PGHS-1 and PGHS-2 (3). We also showed that PGHS-1 and PGHS-2 catalyze the synthesis of malondialdehyde (MDA) and 12-hydroxy-heptadecatrienoic acid (12-HHT), with MDA being a generally accepted biomarker of oxidative stress.

Paradoxically, we found in the same study that reduced glutathione (GSH), the most abundant intracellular low-molecular-mass antioxidant, promotes the formation of 8-*iso*-PGF$_{2\alpha}$, MDA, and 12-HHT (3).

Can we distinguish enzymatic from non-enzymatic sources of PGF$_{2\alpha}$, 8-*iso*-PGF$_{2\alpha}$, MDA, and 12-HHT?

Generally, the proportion of enzymatic and non-enzymatic pathways contributing to PGF$_{2\alpha}$, 8-*iso*-PGF$_{2\alpha}$, MDA, and 12-HHT is unknown and is likely to vary greatly in biological systems. Alternative approaches to distinguish enzymatic and non-enzymatic pathways have been proposed. They involve the use of the molar ratio of 8-*iso*-PGF$_{2\alpha}$ to another prostanoid such as the parent molecule arachidonic acid (6) or prostaglandin E$_2$ (PGE$_2$) (3, 7) (Figure. 1). PGE$_2$ is arbitrarily assumed to being exclusively produced from arachidonic acid by PGHS. Yet, the exclusive source of PGHS for PGE$_2$ remains to be demonstrated. Noteworthy, 8-*iso*-PGE$_2$ is known to readily and spontaneously isomerize to PGE$_2$, so that the origin of PGE$_2$ and its metabolites measured in biological samples are eventually uncertain. This may also apply to prostacyclin (PGI$_2$) and thromboxane A$_2$ (TxA$_2$) (8). These issues underline the Gordian nature of oxidative stress.

Analytical and methodological issues contributing to uncertainty and controversies

Two important issues of the proposal by van't Erve et al. (5) are worthy of discussion: (a) The use of different kinds of
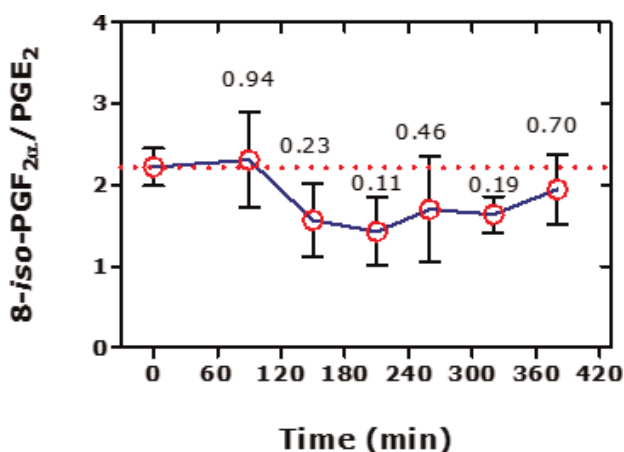

Figure 1. Effects of *N*-acetylcysteine (NAC) on the urinary 15(*S*)-8-*iso*-PGF$_{2\alpha}$/PGE$_2$ molar ratio in eight healthy subjects (4 females, 4 males) upon a single oral intake of 600 mg NAC. NAC was administered immediately after collection of the first urine samples at time 0 h. Urinary excretion rates ranged between 25 and 183 nmol/mol creatinine for 15(*S*)-8-*iso*-PGF$_{2\alpha}$ and between 65 and 315 nmol/mol creatinine for PGE$_2$. Data are shown as mean ± SEM. The numbers indicate the Mann–Whitney test *P* values. This Figure was constructed with data reported in Figure 1 panels (C) and (D) of a previous study (7).

biological samples in which 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ were analyzed and the different pre-analytical and analytical methods applied. (b) The consideration of data obtained under very diverging conditions to distinguish chemical from enzymatic generation of 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ and to quantify the individual contributions.

In general, measurement of primary prostanoids in blood (plasma and serum) is not recommended because of their abundant *ex vivo* formation (discussed in Ref. [9] of this article). In healthy humans, plasma PGF$_{2\alpha}$ concentration ranges between 2 and 11 pg/mL (9). The concentration of free, non-esterified 8-*iso*-PGF$_{2\alpha}$ in plasma of healthy non-smoking volunteers is of the order of 3–10 pg/mL as measured by two highly specific methods, that is, by the combination of immunoaffinity chromatography (IAC) column extraction of 8-*iso*-PGF$_{2\alpha}$ with GC-MS/MS (9). van't Erve et al. reported 5–10 times higher plasma concentrations (range, 50 and 110 pg/mL) for 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ in healthy humans (5). This discrepancy challenges the specificity of the LC-MS/MS methodology as well as the reliability of measuring plasma concentrations of 8-*iso*-PGF$_{2\alpha}$, PGF$_{2\alpha}$, and other primary prostanoids which circulate in blood at concentrations generally below 10 pg/mL (8, 9). The low concentration range is a big analytical challenge for every analytical methodology presently available including GC-MS/MS, but much more so for LC-MS/MS (9). As a consequence of this, the use of molar ratios such as 8-*iso*-PGF$_{2\alpha}$ to PGF$_{2\alpha}$ in plasma is likely to lead to disputable results. Similar difficulties may also apply to other biological samples including urine because of different origin and metabolism of 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ excreted in the urine. In addition, PGHS inhibitors may exert distinctly different effects on 8-*iso*-PGF$_{2\alpha}$ in humans and in widely used experimental animals such as rats (8). Implementation of IAC techniques for the specific separation and isolation of 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ from the remaining potentially interfering 62 F$_2$-prostaglandins prior to LC-MS/MS or GC-MS/MS analysis is recommended.

The input of data obtained under very different conditions into a formula such as that proposed by van't Erve et al. (5) for distinguishing between chemical and enzymatic generation of 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$, as well as for quantifying the relative contribution of the enzymatic and non-enzymatic pathways, is problematic for the following reasons: (a) Data on 8-*iso*-PGF$_{2\alpha}$ obtained from rats and humans are hard to compare (see Ref. [8]). (b) Results obtained from the use of recombinant PGHS enzymes and intact cells are also difficult to compare. (c) The use of the free radicals generating chemical 2,2′-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) at the very high concentration of 10 mM compared to arachidonic acid which was used at the physiologically relevant concentration of about 20 μM is challenging. (d) The very long incubation times (e.g., 5–24 h) in some of the experiments performed by van't Erve et al. (5) may not correctly indicate the actual contribution of chemical and
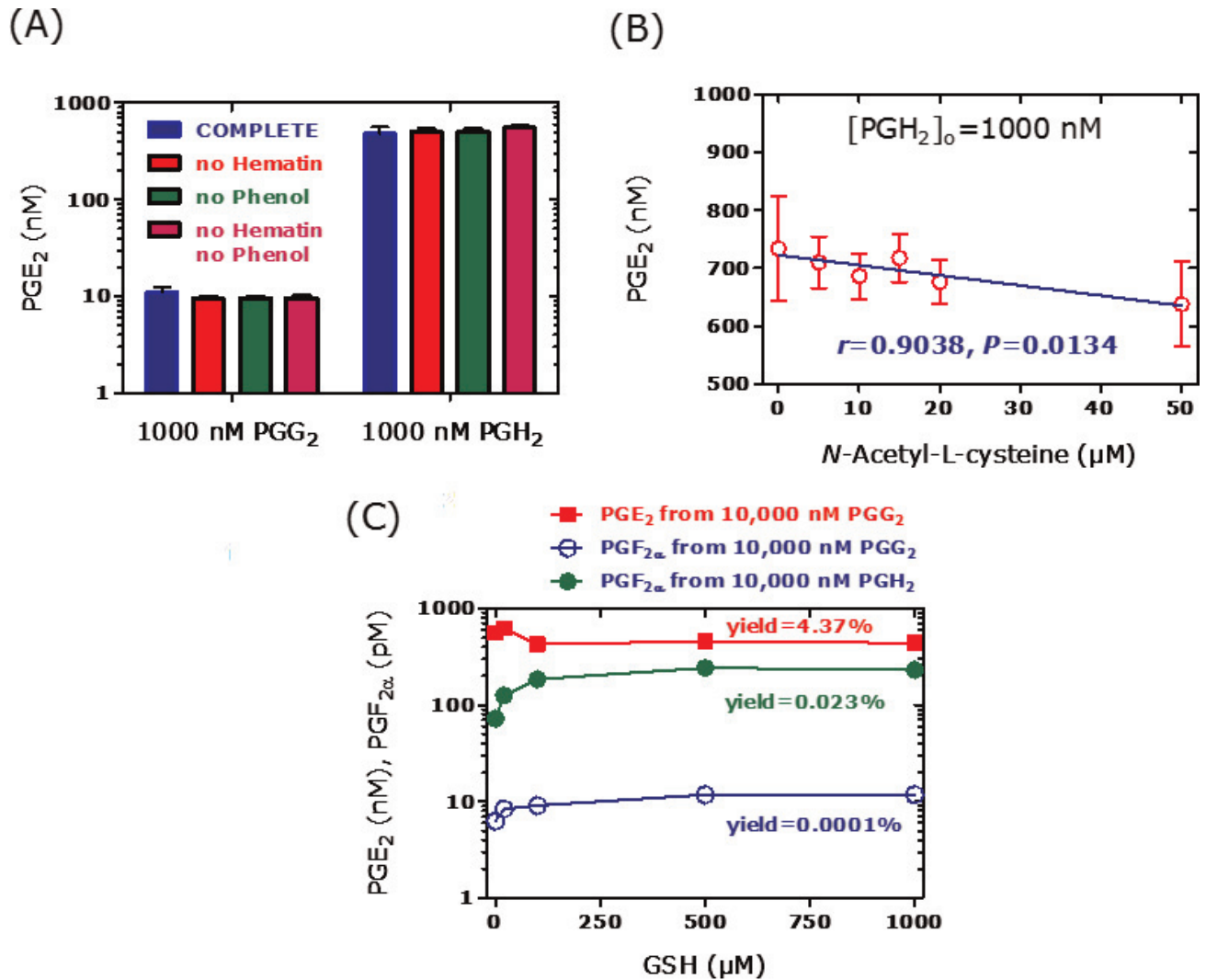
Figure 2. Formation of PGE$_2$ and PGF$_{2\alpha}$ from synthetic PGG$_2$ and PGH$_2$ in buffer at 37°C after incubation for 10 min. (a) Each 1000 nM of PGG$_2$ and PGH$_2$ were incubated in complete buffer (2 mM phenol, 1 µM hematin), as well as in the absence of phenol, of hematin or of both. (b) Effect of various concentrations of *N*-acetyl-L-cysteine (0–50 µM) on the formation of PGE$_2$ from synthetic PGH$_2$ used at a nominal concentration of 1000 nM in complete buffer. (c) Effect of reduced glutathione (GSH) used at various concentrations (0–1000 µM) on the formation of PGE$_2$ from synthetic PGG$_2$ and PGH$_2$ used at a nominal concentration of 10,000 nM each in complete buffer. Experimental conditions and prostanoids analysis were as described elsewhere (3). Data are shown as mean and standard deviation of two independent experiments.

enzymatic peroxidation of arachidonic acid. Finally, (e) the measurement of 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ in blood is not suitable for the reasons discussed above. With respect to the very long incubation time of at least 5 h, it should be emphasized that "oxidative stress" occurs immediately upon addition of arachidonic acid to PGHS (3) under experimental condi-tions very similar to those used by van't Erve et al. (5). For instance, in the presence of recombinant PGHS-1 or PGHS-2 and arachidonic acid (10 µM), the thiols glutathione (100 µM) or L-cysteine (100 µM) are rapidly oxidized to their disulfides in a stoichiometry of about 1:1 with respect to

arachidonic acid, and the reaction is already completed after about 20–30 min of incubation at 37°C (3).

PGE$_2$ is the major reaction product that is generated from arachidonic acid by recombinant PGHS-1 and PGHS-2 (3). In the absence of thiols, synthetic PGG$_2$ decomposes spontaneously to a minor extent to PGE$_2$ in a manner independent of hematin and phenol (Figure 2A); the latter are recommended and commonly used in activity assays of isolated and recombinant PGHS-1 and PGHS-2. Under the same conditions, synthetic PGH$_2$ decomposes mainly to PGE$_2$ to a much higher extent of 70%–80% (Figure 2A). In the presence
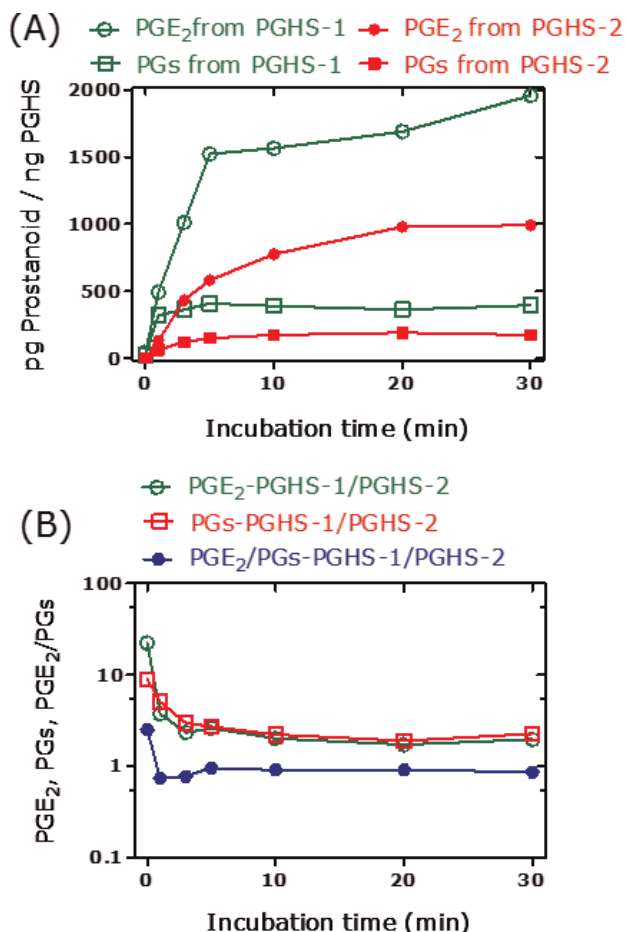
Figure 3. PGE$_2$ and prostanoids (PGs, PGD$_2$+15-keto-PGF$_{2\alpha}$+9ß,11α-PGF$_{2\alpha}$+PGF$_{2\alpha}$+8-*iso*-PGF$_{2\alpha}$) formed in incubation mixtures of arachidonic acid (10 μM), recombinant PGHS-1 (5 U), or recombinant PGHS-2 (5 U) at 37°C for the indicated time points. (a) Amount of PGE$_2$ and PGs per pg PGHS-1 or pg PGHS-2. (b) Molar ratio of PGHS-1- to PGHS-2-derived PGE$_2$, PGs, and their molar ratio. Experimental conditions and prostanoids analysis were as described elsewhere (3).

of the thiol *N*-acetyl-L-cysteine (Figure 2B) or glutathione (Figure 2C), the decomposition of PGH$_2$ to PGE$_2$ decreases, while the formation of F$_2$-prostanoids such as 8-*iso*-PGF$_{2\alpha}$, 9β,11α-PGF$_{2\alpha}$, 15-keto-PGF$_{2\alpha}$, and PGF$_{2\alpha}$ increases several fold. On the contrary, thiols seem not to affect decomposition of synthetic PGG$_2$. Similar effects are also seen in the presence of recombinant PGHS-1 or PGHS-2, yet in the absence of synthetic PGG$_2$ or PGH$_2$, in a manner depending on the incubation time (Figures 3 and 4). These observations indicate that certain changes in experimental conditions may affect significantly the pattern of prostanoids and consequently of their molar ratios (Figure 4). Diverging results are also obtained using PGHS inhibitors with different modes of action such as acetylsalicylic acid (irreversible inhibitor) or diclofenac and indomethacin (both reversible inhibitors) (3).
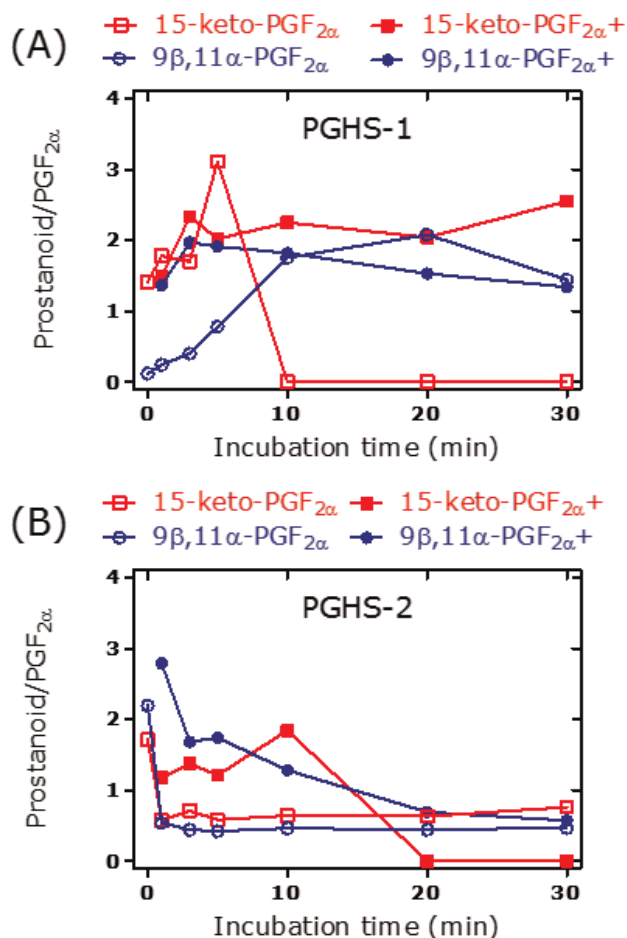
Figure 4. Molar ratio of the prostanoid 15-keto-PGF$_{2\alpha}$ or 9ß,11α-PGF$_{2\alpha}$ to PGF$_{2\alpha}$ in incubation mixtures of arachidonic acid (10 μM) and (a) recombinant PGHS-1 (5 U) or (b) recombinant PGHS-2 (5 U) in the absence or in the presence (indicated by the symbol +) of *N*-acetyl-L-cysteine (100 μM) at 37°C for the indicated time points. Experimental conditions and prostanoids analysis were as described elsewhere (3).

Conclusion

The scientific research area of oxidative stress is highly challenging, and the role attributed to free radicals in the development of chronic diseases and aging process is overestimated (10). Many of the biomarkers being presently used to quantify oxidative stress, including 8-*iso*-PGF$_{2\alpha}$ and MDA, can be generated both by enzymes and free radicals or by non-radical reactive oxygen/nitrogen species, which, in turn, are produced both by enzymes and chemicals especially including transition metal ions. Endogenous substances such as GSH can modulate the fate of intermediates (i.e., PGG$_2$ and PGH$_2$) in favor of 8-*iso*-PGF$_{2\alpha}$ and PGF$_{2\alpha}$ and at the cost of other prostaglandins such as PGE$_2$ and PGD$_{2\alpha}$, the kinetics and yield of reaction products. Distinguishing "enzymatic" from "non-enzymatic" pathways/reactions that lead to 8-*iso*-PGF$_{2\alpha}$, PGF$_{2\alpha}$, and MDA by means of a

combination of *in vitro* and *in vivo* studies is extremely difficult, if not impossible. The focus in this area should be on those F_2-prostaglandins which, in theory, are unlikely to be produced from arachidonic acid by the catalytic action of PGHS and/or of other enzymes/proteins. Some of those F_2-isoprostanes have been sporadically investigated and used as biomarkers of oxidative stress in the past. Studies comparing 8-*iso*-PGF$_{2\alpha}$, a type III (15 series) F_2-isoprostane, with F_2-isoprostanes of type IV (8 series), type V (12 series), or VI (5 series) will provide valuable information about the nature of oxidative stress, notably of lipid peroxidation. COX inhibitors and antioxidants, most notably thiols such as *N*-acetylcysteine, are likely to generate different results *in vitro* using recombinant PGHS enzymes, in cells, and *in vivo* in animals and in humans for many different reasons which have not been fully recognized thus far.

8-*iso*-PGF$_{2\alpha}$ has been recently declared as "the best biomarker of oxidative stress" in humans, and the 8-*iso*-PGF$_{2\alpha}$/PGF$_{2\alpha}$ molar ratio has been proposed as a quantitative measure of lipid peroxidation (5). Yet, scientifically sound evidence has not been provided. The dual nature of oxidative stress and the diprosopus 8-*iso*-PGF$_{2\alpha}$, PGF$_{2\alpha}$, and MDA are likely to be objectives of research of scientist generations to come.

Conflict of interest

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