

# Vitamin C increases the formation of prostacyclin by aortic rings from various species and neutralizes the inhibitory effect of 15-hydroperoxy-arachidonic acid

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- 1 Aortic rings from rats, rabbits and guinea-pigs produce different amounts of 6-oxo-prostaglandin  $F_{1\alpha}$  (6-oxo-PGF $_{1\alpha}$ ), the stable breakdown product of prostacyclin, i.e.  $2760 \pm 195$ ,  $160 \pm 10$  and  $87 \pm 17$  pg 6-oxo-PGF $_{1\alpha}$  per mg wet weight in 30 min.
- 2 Vitamin C enhances the production of 6-oxo-PGF $_{1\alpha}$  by the aortic tissue of these three species, independent of their basal release. This increase was only significant if vitamin C was present in the preincubation as well as in the incubation fluid.
- 3 15-Hydroperoxy-arachidonic acid inhibits the production of 6-oxo-PGF $_{1\alpha}$  ( $IC_{50}$ : 6  $\mu$ M) and this inhibitory effect was completely neutralized by vitamin C.
- 4 The increased production of 6-oxo-PGF $_{1\alpha}$  is not due to an increased release of the substrate arachidonic acid. It is suggested that vitamin C enhances the formation of 6-oxo-PGF $_{1\alpha}$  by protecting the cyclo-oxygenase and PGI-synthase.

## Introduction

The formation of prostacyclin (PGI $_2$ ), which protects the arterial wall against deposition of platelets, is inhibited by hydroperoxides of unsaturated fatty acids (Moncada, Gryglewski, Bunting & Vane, 1976; Salmon, Smith, Flower, Moncada & Vane, 1978). Therefore, it has been suggested that there is a rational basis for evaluating antioxidants on their ability to increase PGI $_2$ -formation by interfering with the formation or the activity of these hydroperoxides (Gryglewski, Bunting, Moncada, Flower & Vane, 1976). Vitamin C has been shown to increase the formation of 6-oxo-PGF $_{1\alpha}$ , the stable breakdown product of PGI $_2$ , in ram seminal vesicle microsomes (Beetens & Herman, 1980; Ravikumar, Pai, Zmijewski & Sih, 1979) as well as in rabbit aortic tissue (Beetens, Van de Bossche & Herman, 1982). In the present study, the effect of vitamin C on the release of 6-oxo-PGF $_{1\alpha}$  by aortic tissue of three different species was evaluated and the mechanism by which vitamin C increased this release was investigated.

## Methods

### *Measurement of endogenous release of 6-oxo-prostaglandin $F_{1\alpha}$ by aortic rings*

Rats (Wistar, males, 200–300 g), guinea-pigs

(males, 300–500 g) and rabbits (males 2.5–3.0 kg) were killed by a blow on the head; the rabbits were then exsanguinated. The thoracic aortae were immediately removed and placed in ice-cold Tris buffer (50 mM, pH 8.3 at 4°C). The aortae were carefully freed from fat and adjacent tissue, and cut into rings of 1 cm. Rat, guinea-pig and rabbit aortae were cut in 2, 3 and 6 pieces, respectively: one piece of each aorta served as control while the other piece(s) were incubated with various concentrations of vitamin C.

The rings were preincubated at 37°C for 15 min in 1 ml Tris buffer (50 mM, pH 7.4), in order to reduce the influence of the manipulation on the PGI $_2$  production. Thereafter, the rings were transferred to 1 ml fresh incubation medium and incubated for another 30 min at 37°C. Each ring was incubated separately. The incubation was stopped by cooling the test tubes on ice, addition of indomethacin (28  $\mu$ M, final concentration) and removal of the tissue. The incubation mixture was assayed for 6-oxo-PGF $_{1\alpha}$  by radioimmunoassay as previously described (Beetens *et al.*, 1982).

A stock solution of vitamin C (10 mg ml $^{-1}$ ) in Tris buffer was freshly prepared for each experiment, and further diluted with buffer to the desired concentration, just before the incubation. 15-HPETE was prepared from arachidonic acid using soybean lipox-

ygenase (Hamberg & Samuelsson, 1967). 15-HPETE was stored in diethyl ether:methanol (9:1) at  $-20^{\circ}\text{C}$ . Before the incubation, the organic solvents were evaporated under a gentle stream of  $\text{N}_2$  and 15-HPETE was redissolved in Tris buffer. 15-HPETE was preincubated for 15 min at  $37^{\circ}\text{C}$ . 15-HPETE was also present in the medium during the incubation period.

*Release of  $1\text{-}^{14}\text{C}$  radioactive material by rat aortae preincubated with  $[1\text{-}^{14}\text{C}]$ -arachidonic acid*

Rats were killed by a blow on the head. The thoracic aortae were immediately removed and rinsed in ice-cold Tris buffer. They were freed from fat and connective tissue, and cut into two rings of 1 cm. The rings were then preincubated for 2 h, at room temperature, in 1 ml Tris buffer containing 700,000 d.p.m.  $[1\text{-}^{14}\text{C}]$ -AA.

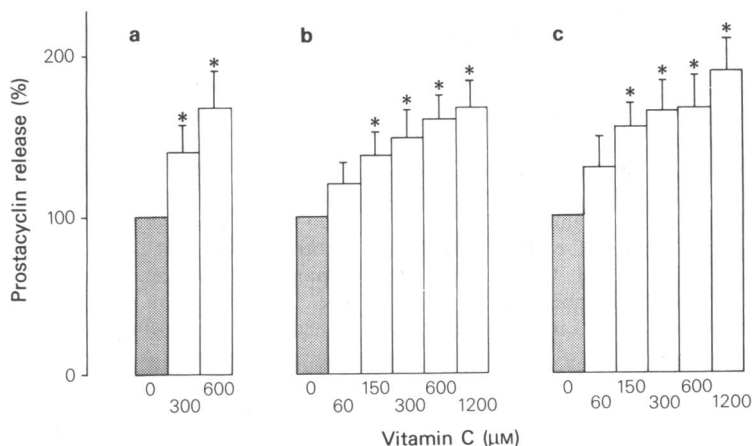
The rings were washed 5 times in Tris buffer at  $0^{\circ}\text{C}$ , for 5 min until the release of radioactive material into the buffer was minimal. The rings were then transferred to test tubes containing 1 ml of Tris and incubated for 30 min at  $37^{\circ}\text{C}$ . The reaction was stopped by cooling the tubes on ice, addition of indomethacin ( $28\text{ }\mu\text{M}$ , final concentration) and removal of the tissue. The wet weight of the rings was determined and they were subsequently dissolved in Soluene-100 (24 h, at room temperature); the radioactivity was determined by liquid scintillation counting.

From the incubation fluid,  $100\text{ }\mu\text{l}$  was transferred to a counting vial for the determination of the total

radioactivity released in the medium. The pH of the remaining incubation fluid was adjusted to 3.0, with citric acid ( $2.3\text{ M}$ ), and the reaction products were extracted twice with 2 ml ethyl acetate. The combined organic phases were dried under nitrogen. The residues were redissolved in  $50\text{ }\mu\text{l}$  chloroform:methanol (2:1, v/v) and spotted on t.l.c. plates together with authentic standards (6-oxo-PGF $_{1\alpha}$ , PGF $_{2\alpha}$ , PGE $_2$ , PGD $_2$ , PGA $_{2\alpha}$ ), AA:  $5\text{ }\mu\text{g}$  of each). The plate was developed in the organic phase of 2,2,4-trimethyl pentane:ethyl acetate:acetic acid:water (50:110:20:100). The different metabolites were located by radiochromatogram scanning, visualized by spraying with phosphomolybdic acid and scraped off. The radioactivity in each zone was quantified by liquid scintillation counting.

*Materials*

Radioactive  $[1\text{-}^{14}\text{C}]$ -arachidonic acid (AA;  $56.5\text{ mCi mmol}^{-1}$ ) and  $[5,8,9,11,12,14,15\text{-}^3\text{H}]$  -6-oxo-prostaglandin  $\text{F}_{1\alpha}$  were purchased from New England Nuclear. Vitamin C was obtained from Roche Co. (Basel, Switzerland). All prostaglandin standards (PGF $_{2\alpha}$ , PGE $_2$ , PGD $_2$ , PGA $_2$  and 6-oxo-PGF $_{1\alpha}$ ) were obtained from Dr J. Pike (Upjohn, Kalamazoo, MI, USA). Thin layer chromatography (t.l.c.) was performed on silica plates 60F $_{254}$  (Merck, Darmstadt, F.R.G.). All solvents used were of analytical grade (Merck). The liquid scintillation fluid, Instagel, and Soluene-100 were purchased from Packard.



**Figure 1** Influence of various concentrations of vitamin C on the release of prostacyclin by aortic tissue of (a) guinea-pig, (b) rabbit and (c) rat. Controls were taken as 100% and the results obtained in the experiments with vitamin C were expressed as a percentage of the control. \*Significantly different from control ( $P < 0.05$ ; Duncan's test,  $n > 6$ ).

## Results

### Basal release of 6-oxo-PGF<sub>1α</sub> by aortic rings

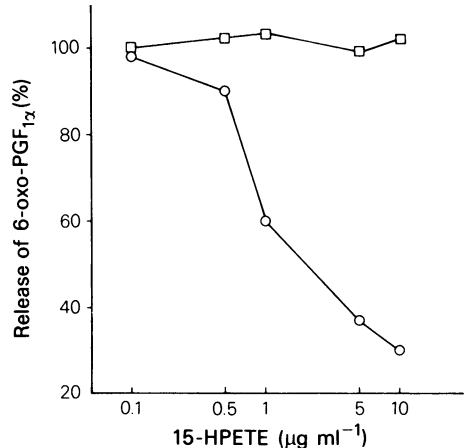
The aortic rings of the three species released different amounts of 6-oxo-PGF<sub>1α</sub>, as calculated on a wet weight basis. Aortic rings of guinea-pigs produced  $87 \pm 17$  pg (mean  $\pm$  s.e. mean,  $n = 9$ ) per mg wet tissue in 30 min. Rabbit aortic rings released  $160 \pm 10$  pg ( $n = 9$ ) and those of rats  $2760 \pm 195$  ( $n = 30$ ). The number of the control rats was greater than those of the guinea-pigs and rabbits, as for each concentration of vitamin C tested, a control group had been taken (see Methods). The basal release of 6-oxo-PGF<sub>1α</sub> by each of the five rat control groups ( $n = 6$  in each group), differed slightly from each other but this difference was never significant (analysis of variance).

### Importance of the presence of vitamin C during the preincubation

To investigate the influence of 'preincubating' the tissue with vitamin C on the release of 6-oxo-PGF<sub>1α</sub>, one series of experiments was set up in which vitamin C (1200  $\mu$ M) was present in the preincubation fluid as well as in the incubation fluid, whereas in another series of experiments, vitamin C was present only during the incubation period. The amount of 6-oxo-PGF<sub>1α</sub> released by the rings preincubated and/or incubated with vitamin C, was compared to the amount released by the control rings. If vitamin C was present only during the incubation period, the formation of 6-oxo-PGF<sub>1α</sub> by rat aorta was slightly but not significantly increased ( $127 \pm 19\%$  of control,  $n = 6$ ,  $t$ test,  $P > 0.05$ ). However, when vitamin C was present during the preincubation period as well as during the incubation period, the increase of 6-oxo-PGF<sub>1α</sub> was much more pronounced ( $190 \pm 15\%$  of control,  $n = 6$ ) and highly significant ( $P < 0.01$ ,  $t$ test). As these experiments clearly show the importance of the presence of vitamin C during the preincubation, in all the following experiments vitamin C was present during both the preincubation and the incubation period.

### Effect of various concentrations of vitamin C on the formation of 6-oxo-PGF<sub>1α</sub>

The effect of various concentrations of vitamin C ranging from 60 to 1200  $\mu$ M, on the production of 6-oxo-PGF<sub>1α</sub> by the aortic rings from the three species, is shown in Figure 1. From 150  $\mu$ M on, vitamin C increased significantly the production of 6-oxo-PGF<sub>1α</sub> in the three species, in a dose-dependent manner.



**Figure 2** Influence of 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (15-HPETE) on the release of 6-oxo-PGF<sub>1α</sub> by rabbit aortic tissue. Results are expressed as a percentage of the control ring, to which no 15-HPETE was added: (O) 15-HPETE; (□) 15-HPETE plus vitamin C. The control for the vitamin C incubations was increased 60% as compared to the other control, due to the effect of vitamin C alone. The concentration of vitamin C was 600  $\mu$ M.

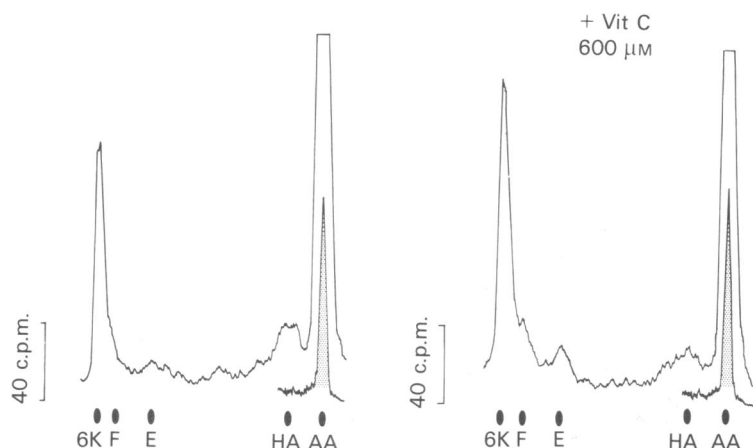
### Influence of 15-HPETE

15-HPETE, a known inhibitor of PGI-synthase, dose-dependently decreased the release of 6-oxo-PGF<sub>1α</sub> by rabbit aortic rings (Figure 2); the IC<sub>50</sub>-value was 6  $\mu$ M. Vitamin C (600  $\mu$ M) completely neutralized the inhibitory effect of 15-HPETE, when they were simultaneously present in the incubation mixture. Indomethacin (28  $\mu$ M) inhibited the formation of 6-oxo-PGF<sub>1α</sub> by more than 90%.

### Release of [1-<sup>14</sup>C]-arachidonic acid and metabolites

Figure 3 shows an example of the metabolites, formed by the rat aortic rings, preincubated with [1-<sup>14</sup>C]-arachidonic acid. The main metabolite was 6-oxo-PGF<sub>1α</sub>; in addition, some hydroxy fatty acids, mainly HHT, and a large amount of unconverted AA, were also present. When vitamin C (600  $\mu$ M) was added to the incubation fluid, the total radioactivity recovered was increased (Table 1). This increase was due mainly to an increased 6-oxo-PGF<sub>1α</sub> production, but the other cyclo-oxygenase metabolites of AA, particularly PGF<sub>2α</sub> and PGE<sub>2</sub>, were also increased. The amount of unconverted AA was not significantly influenced by the presence of vitamin C.

Table 2 shows that vitamin C did not significantly affect the release of AA, when its enzymatic conversion was blocked by indomethacin and eicosa-5,8,11,14-tetraenoic acid. More than 90% of the released



**Figure 3** Radiochromatogram of metabolites of arachidonic acid, released by rat aortic rings, preincubated with [ $^{14}\text{C}$ ]-arachidonic acid (see Methods). The main metabolite is 6-oxo-PGF $_{1\alpha}$ . 6K:6-oxo-PGF $_{1\alpha}$ ; F:PGF $_{2\alpha}$ ; E:PGE $_2$ ; HA: hydroxy fatty acids; AA: arachidonic acid.

radioactivity was free unconverted AA. The higher amount of radioactivity released in these experiments as compared to those previously described can be explained by the presence of albumin in the incubation mixture, which prevents the reuptake of AA released during the incubation period.

## Discussion

It has been shown that vitamin C increases the production of 6-oxo-PGF $_{1\alpha}$  in rabbit aortic rings (Beetens *et al.*, 1982). The results obtained in the present investigation show that this increase is not a species-related phenomenon, and also that the degree of stimulation is independent of the basal release of 6-oxo-PGF $_{1\alpha}$ .

In our experimental conditions, rat aortic rings manifested a higher capacity to release PGI $_2$  than rings of either rabbit or guinea-pig; this has also been reported by other investigators (Hornstra, Hadde-

man & Don, 1978; Bult & Herman, 1979; Tschopp & Baumgartner, 1981).

Indomethacin, an inhibitor of the cyclo-oxygenase, as well as 15-HPETE, an inhibitor of the PGI-synthase, decreased the formation of 6-oxo-PGF $_{1\alpha}$  by the aortic rings, indicating that its precursor, i.e. PGI $_2$ , is enzymatically formed during the incubation period, and that the amount of 6-oxo-PGF $_{1\alpha}$  formed reflects, at least partially, the production of PGI $_2$ .

Vitamin C increased significantly the release of 6-oxo-PGF $_{1\alpha}$ , in a dose-dependent manner. Although the aortic rings of the three species showed a significant difference in basal 6-oxo-PGF $_{1\alpha}$  production, the percentage increase in 6-oxo-PGF $_{1\alpha}$  formation induced by a particular concentration of vitamin C was comparable.

The experiments in which the importance of the presence of vitamin C during the preincubation was investigated, showed that there is a certain delay before vitamin C can optimally increase the production of 6-oxo-PGF $_{1\alpha}$ . This delay probably reflects the

**Table 1** Influence of vitamin C (600  $\mu\text{M}$ ) on the release of [ $^{14}\text{C}$ ]-arachidonic acid and its metabolites by rat aortic rings

	Total radioactivity released		Arachidonic acid		6-oxo-PGF $_{1\alpha}$		Other cyclo-oxygenase products (PGF $_{2\alpha}$ , PGE $_2$ , PGD $_2$ , HHT)	
	d.p.m.	%	d.p.m.	%	d.p.m.	%	d.p.m.	%
Control	29015 $\pm$ 1849		18285 $\pm$ 1984		2971 $\pm$ 568		3854 $\pm$ 263	
Vitamin C	35455 $\pm$ 2913	122 $\pm$ 9*	18746 $\pm$ 2401	102 $\pm$ 8	5121 $\pm$ 867*	178 $\pm$ 8*	6439 $\pm$ 979*	162 $\pm$ 15*

\* $P < 0.05$  (Student's *t* test,  $n = 7$ ), significantly different from control.

Experiments were carried out as described in Methods.

d.p.m.: radioactivity is expressed as d.p.m. per 10 mg tissue. %: the amount of radioactivity found in the incubations with vitamin C was expressed as a percentage of the radioactivity found in the control experiment (paired control).

By this method, the differences were more pronounced.

**Table 2** Influence of vitamin C (600  $\mu$ M) on the release of [ $^{14}$ C]-arachidonic acid by rat aortic rings

	Total radioactivity released		Arachidonic acid	
	d.p.m.	%	d.p.m.	%
Control	69570 $\pm$ 7330		63134 $\pm$ 5935	
Vitamin C	63590 $\pm$ 9530	97 $\pm$ 5	59231 $\pm$ 7438	96 $\pm$ 8

Experiments were carried out as described in Methods. Washing solution as well as the incubation mixture contained indomethacin and ETYA, to block the enzymatic conversion of arachidonic acid. The incubation fluid contained albumin, in order to trap the free arachidonic acid and inhibit its reacylation. Results are expressed as radioactivity released per 10 mg tissue (d.p.m.), or as a percentage of the control (%). The differences were never significant ( $P > 0.05$ ,  $n = 4$ , Student's *t*-test).

need for vitamin C to enter the cell before it can act near the site of the PGI<sub>2</sub> formation. High doses of vitamin C have been shown to increase the release of arachidonic acid and its conversion to prostaglandins in cultures of lung fibroblasts (Polgar, Douglas, Terracio & Taylor, 1980). Our experiments with [ $^{14}$ C]-AA, clearly demonstrate that low doses of vitamin C do not increase the release of the substrate arachidonic acid, but enhance the conversion of arachidonic acid to the metabolite 6-oxo-PGF<sub>1 $\alpha$</sub> . Polgar & Taylor (1980) demonstrated also that this stimulatory effect of vitamin C occurred through hydrogen peroxide formation.

Since vitamin C has been shown in ram seminal vesicle microsomes to reduce the inactivation of the PGI-synthase by the oxidative species, released during the conversion of PGG<sub>2</sub> to PGH<sub>2</sub> (Beetens & Herman, 1982, unpublished) and which is also destructive for the cyclo-oxygenase (Egan, Paxton & Kuehl, 1976), it seems likely that in our experiments with the aortic rings, vitamin C enhances the formation of 6-oxo-PGF<sub>1 $\alpha$</sub>  by protecting both enzymes involved in its biosynthesis, i.e. cyclo-oxygenase and PGI-synthase. The protective effect of vitamin C on the cyclo-oxygenase is further evidenced by an increase in the formation of PGF<sub>2 $\alpha$</sub> , PGE<sub>2</sub> and PGD<sub>2</sub>.

The discrepancy as related to the release of AA between our results and those obtained by Polgar *et al.* (1980), can be explained by the fact that not all cell types respond in the same way to vitamin C. Indeed, the cultures of endothelial or epithelial cells, high doses of vitamin C inhibit the formation of prostaglandins (Polgar *et al.*, 1980). Furthermore, the concentrations of vitamin C used in our experiments were about 10 to 100 times lower than those used by Polgar *et al.* (1980), i.e. 15 mM vitamin C. With lower

doses of vitamin C (1 and 5 mM), Polgar & Taylor (1980) also found a moderate increase in prostaglandin production. However, a sharp rise in prostaglandin synthesis (20 times) occurred between 5 and 15 mM vitamin C.

Thus, it seems likely that at low doses, vitamin C increases the formation of prostaglandin by protection of cyclo-oxygenase and PGI-synthase (our results) but that in very high concentrations (15 mM), the prostaglandin-synthesis is increased by another mechanism, possibly an increased release of substrate (Polgar & Taylor, 1980).

Hydroperoxides of fatty acids, e.g. 15-HPETE, inhibit the PGI-synthase (Gryglewski *et al.*, 1976; Salmon *et al.*, 1978). An increased lipid peroxidation is known to occur in the ageing process and during hyperlipidaemia accompanying atherosclerosis. Therefore the influence of vitamin C on the inhibitory activity of 15-HPETE was studied. Vitamin C was able to neutralize completely the inhibitory activity of 15-HPETE, even at a concentration of 15-HPETE that almost completely blocked the PGI-synthase.

Recently, it has been shown by Weiss, Turk & Needleman (1979), that 15-HPETE exerts its inhibitory activity on PGI-synthase by the generation of an oxidative species, probably a hydroxyl radical. Vitamin C has been shown to be a potent hydroxyl radical scavenger (Bielski, Richter & Chan, 1975). Therefore, vitamin C most probably protects the PGI-synthase against the inhibitory activity of 15-HPETE through the neutralization of this oxidative species.

Our experiments clearly demonstrate that vitamin C is not only able to increase the formation of PGI<sub>2</sub> by the vascular wall but that it also protects the PGI-synthase against the inhibitory activity of hydroperoxides of unsaturated fatty acids. Vitamin C has been reported to have beneficial effects in the treatment of certain vascular disorders (Spittle & Path, 1974). In many experimental studies, high intakes of vitamin C reduce the incidence of atherosclerosis (Turley, West & Horton, 1976), in which PGI<sub>2</sub> production is diminished (Dembinska-Kiec, Gryglewska, Zmuda & Gryglewski, 1977; Sinzinger, Feigl, Silberbauer, Oppolzer, Winter & Auerswald, 1980).

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