

Short Communication

Green tea extract selectively activates peroxisome proliferator-activated receptor β/δ in cultured cardiomyocytes

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(Received 4 June 2008 – Revised 10 September 2008 – Accepted 29 September 2008 – First published online 5 December 2008)

Hypoxia/reoxygenation is one of the causes of the increased expression of inducible NO synthase in cardiomyocytes. In a recent study we demonstrated that a single, high dose of green tea extract (GT) supplemented to the medium of cultured cardiomyocytes just before hypoxia/reoxygenation is able to prevent the increased expression of inducible NO synthase, therefore reducing NO overproduction. In the present study we investigated the mechanism by which GT reduces NO production. Since a molecular mechanism for polyphenol activity has been postulated, and PPAR activation is related to the transcription of the inducible NO synthase gene, we evaluated the activation of PPAR by GT. A moderate GT concentration, supplemented to the cardiomyocyte medium since the initial seeding, selectively activated the PPAR- β/δ isoform. Furthermore, we observed a reduction in NO production and an increase in total antioxidant activity, indicating that GT components may act on both reactive oxygen species, *via* an antioxidant mechanism, and NO overproduction. PPAR- β/δ activation could represent the key event in the reduction of NO production by GT. Although PPAR activation by GT was lower than activation by fenofibrate, it is very interesting to note that it was selective for the β/δ isoform, at least in neonatal cardiomyocytes.

Green tea: Peroxisome proliferator-activated receptors: Neonatal cardiomyocytes: Nitric oxide: Antioxidant activity

Tea, a preparation made from the dried leaves of *Camelia sinensis*, is a popular beverage all over the world; consequently, the significance of daily tea consumption and its possible healthy effects in humans are important issues. Most of tea's properties have been ascribed to its polyphenols, mainly catechins, which have been reported to possess antioxidant, anti-inflammatory and anti-cancer activity^(1,2).

Although promising experimental and clinical data demonstrated protective effects for the cardiovascular system, little is known about the mechanism of action of tea polyphenols at cellular and molecular levels. Evidence is accumulating that tea polyphenols can interfere with multiple pathways of signal transduction in cardiovascular relevant cells, and the induction of multiple effects may play crucial roles in the prevention and treatment of CVD⁽³⁾. The prevention of cardiovascular diseases and reduction of their mortality and morbidity remain some of the greatest public health challenges throughout the Western world.

It has been demonstrated that under pathophysiological conditions such as hypoxia with subsequent reperfusion, when the circumstances allow the formation of substantial amounts of reactive oxygen species and NO, these molecules react

avidly and reactive nitrogen species are generated, causing damage to cellular components⁽⁴⁾.

In a recent study⁽⁵⁾, we demonstrated that the supplementation with a high concentration (about 150 μM -epigallocatechin-3-gallate (EGCG)) of a green tea extract (GT) to the medium of cultured cardiomyocytes just before the induction of hypoxia/reoxygenation is able to prevent the increase of inducible NO synthase expression. This could reduce NO overproduction; let us hypothesise that GT components act on both reactive oxygen species, *via* an antioxidant mechanism, and NO.

In the present study we investigated the mechanism by which GT reduces NO overproduction. Since a molecular mechanism for polyphenol activity has been postulated⁽⁶⁾, and PPAR activation is related to inducible NO synthase expression⁽⁷⁾, we evaluated the activation of PPAR by GT.

In order to verify the effect of chronic exposure to a moderate dose of GT, cardiomyocytes were grown since the initial seeding in a medium supplemented with a GT concentration corresponding to the EGCG plasma level related to the consumption of about five cups of green tea (about 30 μM -EGCG). After hypoxia/reoxygenation, NO production and total antioxidant activity were also measured.

Abbreviations: EGCG, epigallocatechin-3-gallate; GT, green tea extract.

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Materials and methods

Materials

Horse serum, fetal calf serum, Ham F10, fenofibrate and other biochemicals were from Sigma Chemical Co. (St Louis, MO, USA). PPAR transcription factor assay kits were purchased from Cayman Chemicals (Ann Arbor, MI, USA). GT was from Indena (Milan, Italy) and was defined by the producer as having a polyphenol content of $75 \pm 5\%$ (w/w), an EGCG content of $30 \pm 5\%$ (w/w), a content of other catechins of $40 \pm 10\%$ (w/w) and a caffeine content $<8\%$ (w/w) (by HPLC). GT was dissolved in warm bi-distilled water at the concentration of 1 mg/ml and kept at 4°C.

Methods

Primary heart cells were obtained from Wistar rats, aged 2–4 d, as reported⁽⁸⁾, and grown until confluence in control medium (Ham F10 plus 10% fetal calf serum plus 10% horse serum) or in the same medium supplemented with GT (50 μ g/ml). Media were changed every 2 d; the last change was 48 h before the experiment. Hypoxia was obtained by transferring cells to an air-tight, thermostated chamber (Bug Box; Ruskin Technology Ltd, Leeds, West Yorks, UK), as reported⁽⁸⁾. The hypoxia experiments lasted for 2 h, followed by 15 min reoxygenation. Then culture medium was removed, and NO production was evaluated in the medium using the Griess reaction after the construction of a standard curve by the use of known concentrations of sodium nitrite⁽⁹⁾.

Cells were washed three times in PBS, and scraped off. Protein concentration was determined in each culture plate⁽¹⁰⁾ to be sure that variables under study could not be affected by cell number. Cytosolic and nuclear fractions were separated by differential centrifugation⁽¹¹⁾. Total antioxidant activity was determined in the cytosol by the method of Re *et al.*⁽¹²⁾. PPAR activation was determined by ELISA utilising the PPAR transcription factor assay kits (Cayman Chemicals), following the manufacturer's instructions. Briefly, a specific double-stranded DNA sequence containing the peroxisome proliferator responsive element is immobilised onto the bottom of wells of ninety-six-well plates. Samples of nuclear extracts diluted to obtain 50 μ g protein in each assay were added in each well, so that PPAR contained in the extract bind specifically to the peroxisome proliferator responsive element. Different PPAR isoforms were detected by the addition of specific primary antibodies directed against PPAR- α , - β/δ or - γ . A secondary antibody conjugated to horseradish peroxidase was added to provide a sensitive colorimetric readout at 450 nm.

Statistical analysis

Data are presented as mean values and standard deviations. Statistical analysis was by the Student's *t* test.

Results

As previously reported⁽¹³⁾, hypoxia/reoxygenation dramatically increased NO production in control cardiomyocytes (Table 1). GT supplementation significantly reduced NO overproduction,

Table 1. NO production and cytosolic total antioxidant activity (TAA) in control and green tea extract (GT)-supplemented cardiomyocytes, in basal conditions and after hypoxia/reoxygenation (H/R) (Mean values and standard deviations)

	NO (mmol/ml)		TAA (trolox equivalents/ml)	
	Mean	SD	Mean	SD
Control normoxic	38.45	8.39	86.03	4.54
GT normoxic	29.84	5.21	85.60	5.41
Control H/R	582.15***	50.55	80.86*	4.88
GT H/R	148.92**	37.31	98.64***	1.81

Mean value was significantly different from that of the control normoxic cardiomyocytes: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

according to the previously reported inhibition of inducible NO synthase overexpression⁽⁵⁾.

After hypoxia/reoxygenation, as a consequence of NO and free radical overproduction, cytosolic antioxidant defences significantly decreased in control cardiomyocytes, while in GT-supplemented cardiomyocytes cytosolic total antioxidant activity appeared significantly higher than in basal, control cells (Table 1).

The nuclear extract derived from each sample was used to simultaneously evaluate the activation of all PPAR isoforms. Results obtained were expressed as the percentage of the value in control cells in basal conditions, considered as 100%.

PPAR- α and PPAR- γ activation was not influenced either by hypoxia/reoxygenation or GT supplementation (data not shown). On the contrary, GT supplementation caused a significant PPAR- β/δ activation, particularly evident after hypoxia/reoxygenation (Fig. 1). In order to better quantify the activation induced by GT on this PPAR isoform, in some experiments cells in basal conditions were stimulated for 4 h with 10 μ M-fenofibrate, a pharmacological agent that ligands and activates PPAR. Either GT or fibrate significantly activated PPAR- β/δ (GT 115.12 (SD 3.49) %; fibrate 188.95 (SD 8.72) % of control; $P < 0.001$), although the effect of the pharmacological molecule was more evident.

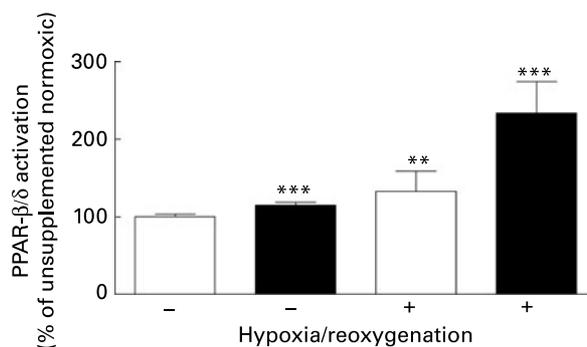


Fig. 1. PPAR- β/δ activation in the nuclear extract of control (□) and green tea extract-supplemented (■) cardiomyocytes, in basal conditions (-) and after hypoxia/reoxygenation (+). PPAR- β/δ activation is expressed as a percentage of the value in control cells in basal conditions, assigned as 100%. Data are means of at least six samples obtained in different cell cultures, with standard deviations represented by vertical bars. Mean value was significantly different from that of the control cells in basal conditions: ** $P < 0.01$, *** $P < 0.001$ (Student's *t* test).

Discussion

In the past decade, substantial progress has been made concerning our knowledge of bioactive components in plant foods and their links to health. Vegetable foods contain many hundreds of compounds which cannot be considered as nutrients, but appear to play a role in the maintenance of health. Bioactive components of edible vegetables include polyphenols, which display antioxidant properties. However, the bioavailability of potential antioxidants from plant foods is generally too low to have any substantial direct effect on reactive oxygen species, and it is postulated that the mechanism of action of polyphenols, even in very low concentrations, might entail their interaction with cell signalling and might influence gene expression, with the consequent modulation of several cell activities⁽¹⁴⁾.

The present results indicate that a moderate dietary concentration of GT prevents NO overproduction and increases antioxidant defences, as indicated by cytosolic total antioxidant activity. The final result may be a counteraction of oxidative damage, which is the result of an imbalance between pro-oxidant and antioxidant molecules. GT appears to protect cells by reducing the former while increasing the latter.

Since it is conceivable that NO overproduction is prevented via the previously reported reduction of hypoxia/reoxygenation-induced inducible NO synthase overexpression, and Paukkeri *et al.*⁽⁷⁾ recently demonstrated that PPAR agonists reduce lipopolysaccharide-induced inducible NO synthase expression and NO production in macrophages, we measured the effect of GT on the activation of the different PPAR isoforms.

GT supplementation had no effect on the α and γ isoforms, while it significantly activated the β/δ one, in both basal conditions and after hypoxia/reoxygenation. PPAR activation by GT was lower than activation by fenofibrate, conceivable considering that fibrates are pharmacological agents.

Although both PPAR- α and - γ are expressed in neonatal rat ventricular myocytes⁽¹⁵⁾, PPAR- β/δ is the predominant PPAR subtype in this cell type and plays a prominent role in the regulation of cardiac lipid metabolism⁽¹⁶⁾. It has been reported that PPAR- β/δ activation inhibits lipopolysaccharide-induced NF- κ B activation⁽¹⁶⁾. Since activation of the transcription factor NF- κ B facilitates the transcription of a number of genes such as inducible NO synthase⁽¹⁷⁾, activation of PPAR- β/δ could represent the key event in the reduction of NO production by GT.

At present, it is difficult to distinguish whether PPAR activation by GT is due to direct catechin binding to the transcription factor or it is caused by an increased availability of PPAR- β/δ . EGCG has been reported to inhibit proteasomal activity⁽¹⁸⁾, which, in turn, regulates the availability of transcription factors such as PPAR⁽¹⁹⁾. Notwithstanding, it is very interesting to note that GT selectively activated the β/δ isoform, at least in neonatal cardiomyocytes.

Further studies are needed to better understand the molecular effects of GT in cardiomyocytes, but data reported in the present study appear promising, not only because it is demonstrated that an aberrant NO production has detrimental consequences and is involved in the pathophysiology of myocardial infarction⁽²⁰⁾, but also considering the possible effect of GT as a PPAR- β/δ selective activator. Very recently, Sheng *et al.*⁽²¹⁾

demonstrated that GW0742, a synthetic selective agonist of this PPAR isoform, exerts a beneficial effect on angiotensin II-induced cardiac hypertrophy. In our knowledge there are no data in the literature regarding PPAR activation by green tea, apart from the study by Lee⁽²²⁾ on mouse cloned PPAR- α . Other studies are needed to verify if GT selective activation of PPAR- β/δ is a feature limited to neonatal rat cardiomyocytes or more generally to cardiac cells. Maybe in the near future modern medicine will be able to better explain why tea was considered the 'long-life beverage' by ancient medicine.

Acknowledgements

The present study was partially supported by the Centro Interdipartimentale di Ricerca sull'Alimentazione Umana, University of Bologna (Italy).

M. D. N. and E. B. performed the analysis together with F. D., who also did the data analysis and contributed to the drafting of the paper; A. B. designed and supervised the study, and wrote the paper.

All authors state that there is no conflict of interest associated with the present study.

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