Electrochimica Acta 240 (2017) 466–473

Contents lists available at ScienceDirect

Electrochimica Acta

journal homepage: www.elsevier.com/locate/electacta

Anti- and pro-oxidative activity measured directly as the extent of 8-oxoguanine production

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ARTICLE INFO

Article history:
Received 21 January 2017
Received in revised form 11 April 2017
Accepted 15 April 2017
Available online 18 April 2017

Keywords:
Antioxidant activity
8-oxoguanine
polyphenolics
ROS
voltammetry

ABSTRACT

An electrochemical method was developed for the evaluation of antioxidant activity based on the ability to inhibit 8-oxoguanine production. It consists of a sequence of successive potential hold and scan steps, in which superoxide anion radical is generated by the reduction of dioxygen in a potential hold step in a buffer solution containing guanine and antioxidant, immediately followed by a potential scan step, in which 8-oxoguanine is detected and its amount determined. The effect of 11 antioxidants, among them simple alcohols for comparison, was investigated in pH 7 and pH 9 phosphate buffers saturated with guanine. The plots of 8-oxoguanine peak height, expressed as percent of the peak current in the absence of antioxidants, versus antioxidant concentration showed an exponential decay character. The decay parameter may be used as a measure of antioxidant activity. It is directly related to the IC_{50} coefficient used in other methods Only simple alcohols displayed single decay character. Other studied substances showed multi-exponential decay at pH 7, most investigated antioxidants were unable to entirely inhibit the generation of 8-oxoguanine, but led to a steady state of 8-oxoguanine level. At pH 9, many antioxidants, particularly polyphenolics and ascorbyl phosphate, showed pure pro-oxidant activity.

1. Introduction

8-Oxoguanine (7,8-dihydro-8-oxoguanine) is the most frequently used marker of oxidative DNA damage [1]. It is a product of $2e^-/2H^+$ oxidation of guanine, the DNA base having the lowest oxidation potential and ionisation energy [2], and hence, being the most susceptible to oxidation. DNA lesions caused by oxidative stress are often the primary cause of many human diseases and aging [3]. They are a consequence of mutations initiated by mispairing of 8-oxoguanine with thymine instead of cytosine, as guanine should [4]. Recently, even neurodegenerative disorders were linked to the accumulation of 8-oxoguanine in mitochondrial DNA in brain cells [5]. Most of these lesions start with uncontrolled release of the product of 1-electron dioxygen reduction, superoxide anion radical, $O_2^{\bullet}-$. This might happen in mitochondrial respiratory chain, owing to leaking electrons, particularly from Complex III [6], but quite purposefully in immune cells, where dioxygen is reduced to $O_2^{\bullet}-$ by NADH to fight attacking pathogens. Superoxide anion radical is rapidly protonated yielding hydroperoxide radical, HO_2, that may undergo either further reduction or dismutation giving hydrogen peroxide, H_2O_2. These compounds and some highly reactive products they may be transformed to are jointly called Reactive Oxygen Species (ROS).

The problem with detecting 8-oxogunine as a product of guanine oxidation is that 8-oxoguanine undergoes oxidation at potentials by ca. 0.3 V less anodic than guanine [7], thus it will be further oxidised at the potential at which it is formed yielding a couple of products, like spiroiminodihydantoin and guanidinohydantoin [8]. Moreover, both guanine and 8-oxoguanine oxidation are $2e^-/2H^+$ processes, which means that the potential will shift cathodically by 59 mV per pH unit with increasing alkalinity, but the difference will remain the same (up to pH 11 [9]). However, 8-oxoguanine is a product of a consecutive reaction that requires some time to occur. If the scan rate is sufficiently high and the potential scan is reversed at a correct moment, then, the oxidation wave of 8-oxoguanine will be seen in the subsequent forward scan [10]. Sometimes, it may accumulate on the carbon electrode and its oxidation wave may be seen in repeated scans after a couple of scans [11].

The formation of 8-oxoguanine and, consequently, the mutation, may be inhibited by adding antioxidants, species that would competitively react with ROS, and hence protect against oxidative

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http://dx.doi.org/10.1016/j.electacta.2017.04.074
0013-4686/

stress. Electrochemical methods are often used for evaluation of antioxidative activity, among them methods based on the reactivity towards superoxide anion in DMF [12] or just the determination of redox potential of antioxidants to show their potential ability to react with ROS. Recently, the degree of protection against oxidation by various ROS of guanine deposited on the electrode was employed as an indicator of antioxidative activity [13–15]. It was expressed as the ability to restore the intensity of guanine anodic peak current in cyclic voltammetry or square wave voltammetry. In these measurements, no 8-oxoguanine was detected. As free radicals, among them some ROS, are responsible for the oxidation of DNA bases, the ability to effectively react with radicals was also used for their evaluation [16]. Antioxidant content of foods became a highly sought-after parameter, so that a need emerged for adopting a standardised total antioxidant parameter for use as a nutritional index for food and biological fluids [17–19].

Substances regarded as antioxidants are able not only to reduce the level of ROS, under some conditions, they are also able to generate them, and act as pro-oxidants. A characteristic example is pyrogallol, used in some antioxidant activity evaluation methods as a source of superoxide anion produced in the autoxidation of this polyphenol [20,21]. Other popular antioxidants also exhibit pro-oxidant properties, like Vitamin C [22], which was recently proven to contribute to the formation of extremely active hydroxyl radicals [23]. Even resveratrol may cause massive oxidative stress in mitochondria [24], however, after reduction by leaking electrons it may also produce a strong antioxidant, molecular H_2 [25], so its role is complex. This pro-oxidant activity is revealed particularly in the presence of various catalytically active metal ions [26]. But this activity, and particularly its damaging effect, was observed mostly in *in vitro* studies. Experiments carried out *in vivo* indicated in most cases otherwise. Interestingly, these compounds give rise to an increase in ROS concentration to cytotoxic levels in tumour cells rather and not in the healthy ones. This means that this effect could also be employed for therapeutic purposes. Macromolecular phenolic substances, like lignin or melanin, also show pro-oxidative activity, which may be beneficial and is used in insect and plant defence as part of their immune response [27]. Such natural phenolic matrices may serve as antioxidants or pro-oxidants and, when needed, they may even exhibit UV-protective functions [28]. It was recently noticed that ROS can even promote longevity [29]. It appeared that the lifespan may be an inverted U-shaped function of the ROS level, with an optimal level most favourable for longevity. These *in vivo* studies have also demonstrated that the impact of antioxidants or pro-oxidants depends strongly on the genotype.

Here, we demonstrate, how the presence of antioxidants affects the oxidation of guanine to 8-oxoguanine, the most popular biomarker of oxidative stress. To this end we developed an electrochemical method consisting of successive potential hold and scan steps, enabling the determination of 8-oxoguanine formed directly after cathodic generation of Reactive Oxygen Species in a solution containing both guanine and antioxidants. The amount of 8-oxoguanine formed decayed exponentially with the concentration of antioxidants. Our method shows that antioxidants may not only inhibit this process but also promote it.

2. Experimental

Measurements were done with a BAS 100B/W electrochemical analyser under air atmosphere in a typical 3-electrode cell with a glassy carbon type K working disk electrode (2.3 mm^2, Mineral, Poland) and platinum wire as the auxiliary. The working electrode was polished with 0.05 μm alumina slurry (Buehler) and sonicated in deionised water prior to each series of measurements. Potentials are quoted against the Ag/AgCl (3 M NaCl) reference electrode. Saturated guanine solutions in phosphate buffers of pH 7 and 9 (0.1 M KH_2PO_4/KOH) were prepared by using sonication (15 min) for dissolving guanine, followed by centrifuging off excess solid material. However, there is a problem with the determination of guanine concentration at pH 7. The value of 39 ± 1 μM (at 25 °C) found in the literature [30] is often quoted, but there are reports on serious problems with the determination of guanine solubility. Measurements based on dissolution at a higher temperature and then bringing to equilibrium at 25 °C in citrate-phosphate buffer revealed that the result depended on the excess used for solubilisation [31], with a higher excess yielding almost 3 times higher value than the mentioned above, but when only a slight excess was used, concentrations close to 40 μM were obtained. From our own experience we know that the solubility of guanine in buffer solutions depends on the type of cations present in it. Potassium cations increase the solubility, most probably because this cation stabilises self-assembled guanine quartets and quadruplexes [32] that could spontaneously be formed in solutions. That is why it is important to give a more detailed description of buffer solutions used. Anyhow, we have decided to keep the solution preparation conditions strictly the same for all the measurements. We assumed that in this case the concentration of the solution in pH 7 buffer will be close to 0.04 mM. The concentration of the solution in pH 9 buffer we estimated by comparing the UV–vis spectra of both solutions and calculated it to be ca. 0.05 mM.

Based on preliminary tests we have decided to employ the following programme of alternating potential hold and scan steps:

1st step – Hold at +400 mV for 15 s (electrode stabilisation)

2nd step – Scan from −200 to +1200 mV at 500 mV s^{-1} (checking for 8-oxoguanine residues)

3rd step – Hold at +1200 mV for 2 s (cleaning the electrode)

4th step – Hold at −1000 mV for 2 s (oxygen reduction – generation of superoxide)

5th step – Scan from −200 to +1500 mV at 500 mV s^{-1} (detecting 8-oxoguanine and guanine)

6th step – Scan from +1500 to −200 mV at 500 mV s^{-1} (reverse scan)

At the beginning, to stabilise the electrode, the whole programme was run several times (usually 8 times) in a saturated solution of guanine in phosphate buffers, until we got identical voltammograms for step 5. If despite repeated scanning, the electrode showed too low activity with 8-oxoguanine waves lower than ca. 0.06 mA cm^{-2}, it was better to clean it again using alumina and sonication. Continuing measurements with an electrode not sufficiently active would result in getting irreproducible results.

Employing this programme allows seeing not only the production of 8-oxoguanine but also checking for its presence before the generation of superoxide. In order to dispel suspicions that after step 3 (oxidation at +1.2 V), 8-oxoguanine may remain on the electrode, we added an additional step of holding the potential at +300 mV for 15 s, and checked for the presence of 8-oxoguanine by scanning the potential in the range −200 mV to +1200 mV. As none was detected, we gave up this step in subsequent measurements.

The antioxidants were added in small, μL portions of stock solutions in phosphate buffers used in experiments. To avoid excessive dilution of the guanine solutions in the cell, the maximum amount added did not exceed 1/3 of the initial volume of guanine solution. Usually, the measurement was started with a more concentrated stock solution, then it was repeated with stock solutions of lower concentrations to cover better the initial range of antioxidant concentrations. The stock solutions had to be prepared freshly because of their tendency to get oxidised, except