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Review article

Electrophilic methyl groups present in the diet ameliorate pathological states induced by reductive and oxidative stress: a hypothesis

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Reductive stress, characterised by an increased NADH:NAD^+ ratio, may be as common and as important a consequence of redox imbalance as oxidative stress. It may also be an important predisposing cause of the generation of reactive oxygen species. Considerable experimental and indirect clinical evidence suggests that protection against reductive stress depends on biomolecules with electrophilic methyl groups (EMG) such as *S*-adenosylmethionine, betaine, carnitine and phosphatidylcholine. Pathological processes leading to reductive stress and their relief by such protective agents is reviewed and the proposed molecular mechanism is outlined. These and other EMG-containing biomolecules are part of the daily diet and may represent an important control system for redox balance.

Reductive and oxidative stress: Dietary electrophilic methyl groups: NADH: Methane: Functional food

Redox imbalance in cells and subcellular structures can lead either to oxidative or to reductive stress. Oxidative stress has been extensively studied for many years and its possible clinical ramifications have been explored in considerable depth. Reductive stress, by contrast, has not been widely recognised. Yet reductive stress is probably both common and of clinical importance: indeed, reductive stress plus oxygen rather than oxidative stress may be the most common mechanism leading to the generation of reactive oxygen species (ROS). One possible link between the two may be the reduction of Fe^{3+} and its liberation from ferritin. The reduced metal could catalyse ROS generation (Jaeschke *et al.* 1992; Stäubli & Boelsterli, 1998). Reductive stress may also be an important preliminary in the post-ischaemic generation of ROS. In a general way reductive stress could inhibit or adversely affect a variety of enzymatic pathways (Fig. 1).

Recognition of reductive stress as a potentially common cause of pathological states raises the question of the nature of protective mechanisms in the same way as recognition of oxidative stress many years ago has led to the study of antioxidants. (Indeed, paradoxically perhaps, agents which prevent reductive stress rather than antioxidants may eventually prove to be the most important protective mechanism against ROS damage.) A review of the literature, theoretical molecular considerations and experiments now in progress point to the key role of electrophilic biomolecules capable of oxidising NADH to NAD^+. The *in vivo* action of those may be analogous to the *in vitro* effect of electron acceptors such as dichlorophenolindophenol and methylene blue (Khan & O'Brien, 1995) or acetoacetate and acetaldehyde (Niknahad *et al.* 1995). They may be assumed to have a positively charged N or S atom in their structure, rendering an adjacent methyl group electron deficient.

Biomolecules which fulfil these conditions include phosphatidylcholine (PC), acetylcholine, and sphingomyelin. These biomolecules have a positively charged N atom which makes the adjoining methyl group electron-deficient. They react *in vitro* with the electron donor sodium benzothiolate in an irreversible redox reaction by the

Abbreviations: EMG, electrophilic methyl groups; PEMT, phosphatidylethanolamine methyl transferase; PC, phosphatidylcholine; ROS, reactive oxygen species; SAM, *S*-adenosylmethionine.

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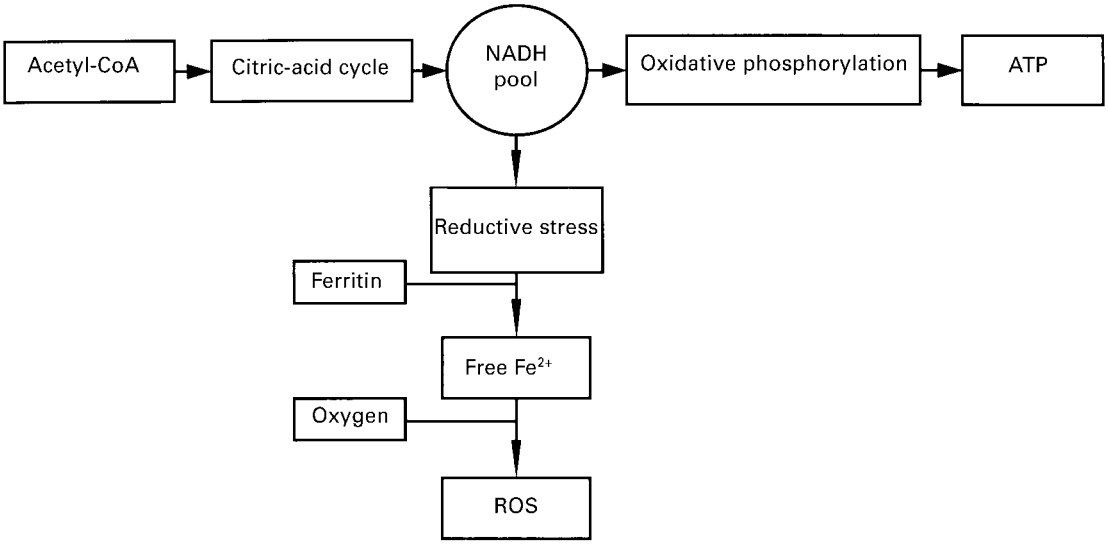

Fig. 1. Schematic illustration of energy conversion, generation of reductive stress and the ensuing formation of reactive oxygen species (ROS).

transfer of a pair of electrons to the electron-deficient methyl group, thus splitting this group from the positive N moiety (Stoffel *et al.* 1971). This electron-pair transfer between the biological electron acceptors and an artificial electron donor led us to speculate that a similar reaction may also take place in animal cells. We hypothesised that biomolecules with a positively charged N or S atom and a bound methyl group also react with biological electron donors such as NADH, thus lowering the high NADH:-NAD^+ ratio. We termed these methyl moieties electrophilic methyl groups (EMG), since they may accept a pair of electrons by virtue of their electron deficiency, which results from the adjoining positively charged N or S centres.

Biomolecules which posses such EMG moiety are *S*-adenosylmethionine (SAM), betaine, carnitine, choline, glycerylphosphocholine, PC, and several other biomolecules which have a trimethylnitrogen moiety. As will be shown later, PC, betaine, carnitine and SAM do indeed ameliorate reductive stress.

There are four different experimental methods for the generation of reductive stress:

1. ethanol intoxication can be followed by the elevation of the lactate:pyruvate ratio (Lieber, 1997);
2. hypoxia can prevent the oxidation of NADH to NAD^+ (Khan & O'Brien, 1995);
3. reductive stress can result from the dislocation of electrons by redox cycling substances such as doxorubicin (Stäubli & Boelsterli, 1998);

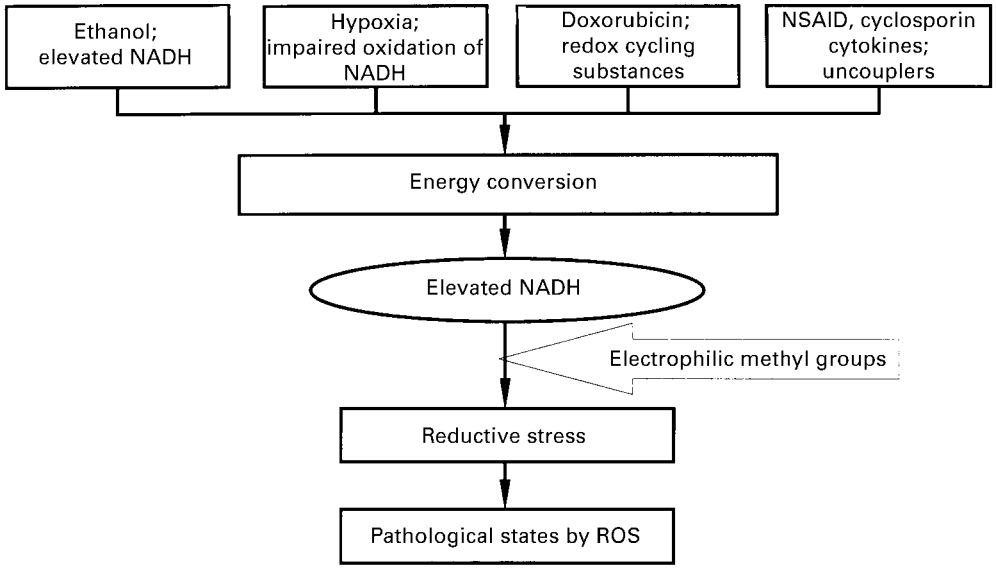

Fig. 2. Generation of reductive stress, the ensuing reactive oxygen species (ROS)-induced damage, and the interception of this pathway by biomolecules with electrophilic methyl groups. NSAID, non-steroidal antiinflammatory drugs.

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4. uncouplers can interrupt the flow of electrons down the electron transport chain (Niknahad *et al.* 1995).

These methods are outlined in Fig. 2, together with the reaction sequences leading to pathological states, and the proposed intercepting mechanism by EMG.

Elevated NADH:NAD^+ ratio induced by ethanol intoxication

The protective effect of PC is documented in baboons maintained on a liquid diet of ethanol or isoenergetic carbohydrate with or without PC supplement for 8 years (Lieber *et al.* 1990*b*). The ethanol-fed animals developed septal fibrosis and cirrhosis, and the transformation of lipocytes into transitional cells occurred in almost all cases. Animals in the PC-supplemented group developed no septal fibrosis, and after discontinuation of PC in the diet progressed to cirrhosis within 18–21 months. In a similar study, baboons were fed a high-ethanol diet with or without the PC supplement (Lieber *et al.* 1994*b*). In the group without the PC supplement nearly all animals developed septal fibrosis or cirrhosis with the transformation of the hepatic lipocytes into the collagen-producing transitional cells. In the group with the PC supplement, the lipocyte transformation was rare, and septal fibrosis and cirrhosis did not develop. In a double-blind, randomised, placebo-controlled trial with patients suffering from ethanolic hepatitis, the survival rate in PC-supplemented group was 69 % as compared with 49 % in the placebo group (Panos *et al.* 1990). Phosphatidylethanolamine methyltransferase (PEMT) plays a key role in the pathway for synthesis of membrane PC. Alcohol feeding significantly decreases PEMT activity in baboons with a corresponding reduction in liver PC levels. It has been demonstrated that a PC-enriched diet ameliorated the ethanol-induced decrease in PEMT activity (Lieber *et al.* 1994*a*). Additionally, PC protected the gastric mucosa in ethanol-induced injury in rats (Szelenyi & Engler, 1986; Dunjic *et al.* 1993). Although PC is not an antioxidant (as antioxidants are chemically defined as electron donors), it prevents CCl_4-induced hepatic lipid peroxidation (Aleynik *et al.* 1997). F2-isoprostanes and 4-hydroxynonenal, breakdown products of lipid peroxidation are significantly increased in baboons fed alcohol, but this was fully prevented by supplementation with 2·8 g PC/4·18 MJ (Lieber *et al.* 1997).

The ameliorating efficacy of betaine in ethanol-induced liver dysfunction is indicated in the following experiments (Barak *et al.* 1993). Rats were maintained for 4 weeks on ethanol and a normal diet in combination with the betaine-lacking or the betaine-containing diet. The betaine administration prevented the formation of fatty liver, and elevated the level of SAM. A follow-up experiment with a similar protocol revealed a dose-dependent efficacy of betaine (Barak *et al.* 1994). In addition, betaine reversed the established steatosis after ethanol challenge was discontinued (Barak *et al.* 1997).

SAM-synthetase and PEMT activities are markedly reduced in human cirrhosis (Duce *et al.* 1988). SAM supplementation reversed the hepatic SAM depletion in baboons fed with ethanol for 15–18 months. In addition, SAM partially prevented the hepatotoxic effect of ethanol (Lieber *et al.* 1990*a*). SAM ameliorated the ethanol-induced liver damage in rats by preserving cellular ATP levels and the mitochondrial membrane potential (Garcia-Ruiz *et al.* 1995). SAM also displayed a protective effect against rat liver steatosis induced by chronic ethanol treatment, and improved recovery from steatosis after ethanol withdrawal (Feo *et al.* 1986). The ameliorating effect of SAM in the damage of gastric mucosa induced by ethanol has also been demonstrated in a study with two groups of healthy human volunteers in which the results were obtained from endoscopic and photographic scores as well as from histopathological samples (Laudanno *et al.* 1987).

In rats, carnitine ameliorated the ethanol-induced fatty liver (Sachan *et al.* 1984) as well as the ethanol- and hypoxia-induced damage (Bertelli *et al.* 1993). The protection was dose-dependent in the chronic alcoholic rats (Rhew & Sachan, 1986). Carnitine inhibited alcohol dehydrogenase (Sachan & Cha, 1994) and the oxidation of ethanol in hepatocytes (Cha & Sachan, 1995).

Impaired NADH oxidation during hypoxia

PC has been shown to have a protective effect in the ischaemic isolated rat heart (Duan & Karmazyn, 1990). When added to an isolated rat heart prior to ischaemia, PC significantly enhanced the chances of recovery, reduced the reperfusion-induced arrhythmia and improved sub-sarcolemmal mitochondrial oxidative phosphorylation. The protective effect of PC was also demonstrated during ischaemia-reperfusion in isolated ventricular tissue (Duan & Moffat, 1990).

SAM ameliorated sequential warm and cold ischaemic injury in rat liver (Dunne *et al.* 1997). SAM was found to protect the liver following addition to both the preservation solution and to the repressing medium, and also when given directly to the donor animal alone. The blood flow increased by 68 % when SAM was used for flushing the preservation solution, and by 58 % when SAM was present throughout the reperfusion. The protective efficacy of SAM was also described in a similar experiment with isolated perfused rat liver (Dunne *et al.* 1994).

Carnitine relieved metabolic changes in man caused by acute tissue hypoxia (Corbucci *et al.* 1992). Patients with an aorto–pulmonary bypass (n 120) were treated with carnitine or placebo. Carnitine was found to normalise the levels of lactate and pyruvate thus ensuring redox balance. It was also found to protect against ischaemia-reperfusion injury of the rat heart. Moreover, in the Langendorff-perfused rat heart, the addition of carnitine decreased the formation of ROS, improved mechanical properties of the heart, prevented the loss in creatine phosphokinase activity, and increased its ATP content (Packer *et al.* 1991). Furthermore, carnitine prevented ischaemia-caused mechanical damage in the diabetic rat heart (Broderick *et al.* 1995). Another study concluded that the beneficial effects of carnitine in ischaemic heart were linked to the inhibition of fatty acid oxidation (Broderick *et al.* 1993).

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Displaced electrons by redox cycling substances

PC significantly reduced acute toxicity of doxorubicin when the latter was administered in association with (Gabizon *et al.* 1986), encapsulated in (Storm *et al.* 1989), or complexed with (Balazsovits *et al.* 1989) PC-based liposomes. Reduced toxicity resulted in prolonged survival, reduced severity of cardiomyopathy and nephropathy (Storm *et al.* 1989), and reduced body and organ weight losses. At a dose of 7·5 mg doxorubicin/kg, 100 % mice receiving liposome-associated doxorubicin survived a cumulative dose of 60 mg/kg administered over 98 d, while 92 % of mice receiving the free drug died (Gabizon *et al.* 1986). In addition, PC-based liposomes significantly decreased oedema, monocytic infiltration, and cellular necrosis (Balazsovits *et al.* 1989). The prerequisite for the improved tolerance was related to the non-specific associations between PC and doxorubicin. This indicates that the presence of PC, and not the liposomes, was the decisive factor for decreased toxicity of doxorubicin.

Uncoupling of the electron chain by non-steroidal antiinflammatory drugs, cyclosporine and cytokines

PC was found to protect the intestinal mucosa in rats against non-steroidal antiinflammatory drugs- and aspirin-induced damage. The effect was independent of the fatty acid composition in the PC molecules, and was documented for different non-steroidal antiinflammatory drugs:PC ratios (Lichtenberger *et al.* 1982; Leyck *et al.* 1985; Szelenyi & Engler, 1986; Soehngen *et al.* 1987; Swarm *et al.* 1987).

PC improved the tolerance of volunteers to cyclosporin A when the two substances were applied as an aerosol. Compared with the formulation containing only cyclosporin A, this mixture diminished tracheal irritation and coughing (Gilbert *et al.* 1997).

SAM was found to antagonise the toxic effect of cyclosporin A in rat hepatocytes (Fernandez *et al.* 1995; Roman *et al.* 1996). In addition, in isolated hepatocytes SAM prevented damage induced by cytokines, such as tumour necrosis factor and interleukin 1. It attenuated the formation of malondialdehyde and lactate dehydrogenase, GSH and impaired triacylglycerol oxidation (Arias-Diaz *et al.* 1996). In addition SAM protected transplanted hepatocytes against the same cytokines (Vara *et al.* 1994).

Carnitine ameliorated the toxic effect of the lipopolysacharide- and methylcholanthreneinduced sarcoma challenges in rats (Winter *et al.* 1995). In these experiments carnitine normalised the levels of interleukin 1β, interleukin 6, tumour necrosis factor and triacylglycerol oxidation, and it exhibited a therapeutic effect on morbidity. In addition, in the epithelial tubular cells of the isolated perfused rat kidney, carnitine was found to attenuate the cyclosporin-induced Ca deposit and enzyme release (Giovannini *et al.* 1996).

Discussion and conclusions

Data from the literature has revealed that the EMG-containing biomolecules ameliorate pathological states induced by reductive stress. The significance of these biomolecules is further emphasised by the fact that they are essential components of the human diet (Blusztajn 1998). A diet without these and related biomolecules, the Lombardi or methyl-deficient diet, generated cancer without the presence of cancerogenic substances (Shinuzoka et al. 1978). Such diets have been the subject of research for more than 50 years but the underlying mechanism is still not understood (Poirier, 1994). Recently, it has been shown in liver mitochondria isolated from rats fed a choline-deficient diet that complex I (NADH dehydrogenase)-linked respiration is impaired coincidentally with alterations in PC metabolism (Hensley, 2000).

In the context of this present paper, it is important to realise that stress induced either by reductive conditions or by an EMG-deficient diet results in identical pathological states. Ethanol-induced reductive stress in rat liver (Garro *et al.* 1991; Lieber, 1997) and an EMG-deprived diet (Poirier, 1994) elicit the same disorders. Both lead to ROS generation, hypomethylation of DNA and impaired oxidation of triacylglycerol. Further indication for the common mode of action is the finding that these pathological states are ameliorated by exogenous EMG-containing biomolecules, as outlined earlier.

The four biomolecules with the EMG-moiety (PC, SAM, betaine and carnitine) differ in their chemical structures and in their currently recognised functions as biomolecules. At the same time they are similar in terms of their EMG groups. They are used as drug substances for similar indications and they are part of man's diet. They form a pool of EMG-containing molecules, which suggests a supply of methyl groups from a common source for a common demand.

Taken together these findings suggest the presence of an endogenous protective system composed of different substances with different chemical structures, but each containing a common chemical moiety. Under normal conditions the pool is in a dynamic balance: EMG are continuously used to maintain redox balance and the pool is continuously replenished by EMG from the diet. However, there are two sets of conditions under which the size of the pool will decrease: (1) pathological reductive stress with the accompanying increased demand for EMG, and (2) pathological deficiency of EMG in the diet. Experiments with the EMG-deficient diets indicate that in human subjects the size of the pool is depressed to a pathological level after 2 weeks (Zeisel *et al.* 1991) and in rats after several days (Poirier, 1994).

The normalisation of the elevated NADH:NAD^+ ratio by EMG and the disappearance of methyl groups by elevated NADH:NAD^+ ratio could be explained by the chemical reaction shown in Fig. 3. In this sequence the nucleophilic hydride ion from NADH is transferred to the EMG from the biomolecule. This is followed by this methyl group splitting off with the formation of CH_4 and the oxidation of NADH to NAD^+. CH_4 is measurable in the breath of approximately one-third of human subjects and is generally considered to be a product of bacterial activity in the gastrointestinal tract. This notion has not been proven unequivocally and there have been several attempts to link the presence of CH_4 in the breath to pathological disorders.

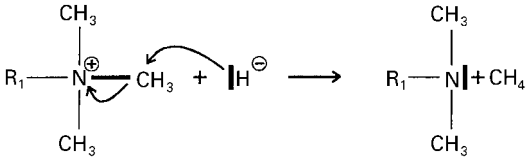

Fig. 3. Proposed chemical reaction for the normalisation of the elevated NADH:NAD^+ ratio by electrophilic methyl groups (EMG). The nucleophilic hydride ion (H^-) from NADH is transferred to the EMG. This is followed by the separation of this methyl group with the formation of CH_4.

This CH_4-generating chemical reaction, and the hypothesis that the methyl group shortage and the CH_4 formation is a marker of reductive stress, is currently under investigation by us.

In conclusion, animal cells normally function in a reductive environment. Various abnormal mechanisms, some of them not uncommon, can transform this into a state of severe reductive stress. This potentially damaging state can be counteracted or perhaps prevented by a protective pool of EMG-containing substances. The protective mechanism involves the consumption of EMG. The state of reductive stress may be an important predisposing cause of ROS generation. We suggest that EMG-containing biomolecules fulfil the proposed criteria for a new class of functional food (Diplock *et al.* 1998, 1999) for the control of redox balance.

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