Hypoxia-Induced Generation of Methane in Mitochondria and Eukaryotic Cells - An Alternative Approach to Methanogenesis

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Key Words

Redox homeostasis • Hypoxia • Phosphatidylcholine
• Oxidative stress

Abstract

Background/Aims: Electrophilic methyl groups bound to positively charged nitrogen moieties may act as electron acceptors, and this mechanism could lead to the generation of methane from choline. The aims were to characterize the methanogenic potential of phosphatidylcholine metabolites, and to define the in vivo relevance of this pathway in hypoxia-induced cellular responses. **Methods:** The postulated reaction was investigated (1) in model chemical experiments, (2) in rat mitochondrial subfractions and (3) in bovine endothelial cell cultures under hypoxic conditions and in the presence of hydroxyl radical generation. The rate of methane formation was determined by gas chromatography with flame-ionisation detectors. The lucigenin-enhanced chemiluminescence assay was used to determine the reactive oxygen species-scavenging capacity of the choline metabolites. **Results:** Significant methane generation was demonstrated in all three series of experiments. Phosphatidylcholine metabolites with alcoholic moiety in the molecule (i.e. choline, N,N-dimethylethanolamine and N-methylethanolamine), inhibited oxygen radical production both in vitro and in vivo, and displayed an effectiveness proportional to the amount of methane generated and the number of methyl groups in the compounds. **Conclusion:** Methane generation occurs in aerobic systems. Phosphatidylcholine metabolites containing both electron donor and acceptor groups may have a function to counteract intracellular oxygen radical production.

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Introduction

Aerobic life depends critically on redox homeostasis, an integrating network of oxidative and reductive processes. A major threat to this controlled equilibrium is hypoxia, when a deficiency of electron acceptor oxygen leads to decreasing ATP generation and progressive functional and structural cell damage [1]. Re-establishment of the molecular oxygen supply is a precarious process, however, as the disturbed intracellular biochemistry leads to the generation of reactive oxygen

species (ROS). Superoxide, a key ROS produced during the incomplete reduction of oxygen forms the basis of "oxidative stress" [2]. This term is applied to *in vivo* situations in which damage is caused, either directly or indirectly, by an elevated level of ROS or the generation of other free radicals.

The major goal of the present work was to outline a hypothetical hypoxia-induced intracellular pathway. In designing our experiments, we took into account the previous finding that the methyl groups of the ubiquitous phosphatidylcholine (PC) molecule are potential electron acceptors [3]. By virtue of the positive charge on the nitrogen atom, these methyl groups may become substitute electron acceptors if there is a deficiency of oxygen, and in consequence of this methane may be liberated [4]. In line with this, we hypothesized that methane generation is a consequence of transient oxygen deprivation in aerobic cells.

We have investigated this hypothesis at three experimental levels: (a) in model chemical reactions with choline and its metabolites in the presence of oxidative stress, (b) in rat liver mitochondria and mitochondrial subfractions under hypoxic conditions, (c) and in endothelial cell cultures with or without oxygen. The results provided evidence that a methane-generating response is induced by hypoxia in aerobic systems, and it may be associated with an intracellular mechanism that affords temporary protection against oxidative stress.

Materials and Methods

Methane-producing capacity of choline metabolites

Test compounds included choline chloride (CC), N,N-dimethylethanolamine (DMEA), N-methylethanolamine (MEA), ethanolamine (EA), betaine, dimethylglycine (DMG), sarcosine (N-methylglycine) and glycine, while DMSO was applied as positive control. The experiments were carried out in 5-ml gas-tight Supelco vials connected to 5-ml syringes through the septum of the cap. The total volume of the reaction mixture was 1 ml and it contained the following components: 1 mM hydrogen peroxide (H_2O_2), 0.5 mM L-ascorbic acid (ASC), 1 mM test compound or dimethylsulfoxide (DMSO), 10 μM FeCl_3 or FeCl_2 and 6 μM EDTA in 5 mM K-phosphate buffer [5]. All chemicals were purchased from Sigma Co. The reaction mixture was adjusted to pH 7.4, the tubes were then closed and air was removed by bubbling nitrogen through the mixture for 20 min. The reaction was started by the addition of H_2O_2 with a Hamilton syringe through the septum of the cap. Any gas mixture generated expanded spontaneously into the syringe. The methane concentration of the gas phase was analyzed in 0.5-ml gas samples by gas chromatography 60 min after initiation of the reaction.

Lucigenin-enhanced chemiluminescence assay

Aliquots of the components were injected into 10-mm-diameter plastic minivials containing lucigenin dissolved in buffer, and the minivials were then placed into Packard potassium glass vials. The total volume of the mixture was 1 ml and it contained 100 μM lucigenin. The resulting chemiluminescence was measured in a liquid scintillation counter (the tritium channel of the Packard Tri-Carb 2100 Model set in the out-of-coincidence mode). The manipulations were performed without external light 2 min after dark adaptation. Changes in chemiluminescence were then detected in the presence or absence of 1 mM test compounds and the results were expressed as percentages of the control values [6].

Experiments with mitochondria

A) Isolation of rat liver mitochondria. Mitochondria were isolated by the method of Schneider as modified by Kristal *et al.* [7]. Mitochondrial protein was determined according to Lowry *et al.* [8]. Mitochondrial integrity and oxygen consumption were analyzed by polarography at 25°C with an oxygraph equipped with a Clark oxygen electrode (Aqueous Phase Respiration System OXYGRAPH/Hansatech Instruments Ltd., UK). Each experiment was repeated with at least five different mitochondrial preparations.

B) Mitochondrial fractions. Mitochondria isolated from rat livers were washed and centrifuged twice with phosphate buffer at 8000 g, suspended in hypotonic 20 mM phosphate buffer (pH 7.4) and centrifuged at 35000 g for 20 min. The supernatant containing the intermembrane space fraction (IMS) was removed. The sediment was then suspended in 10 mM HEPES buffer containing a protease inhibitor cocktail (10 $\mu\text{g}/\text{ml}$ STI, 10 μM leupeptin and 500 U/ml aprotinin) and centrifuged at 1900 g for 25 min. The supernatant was centrifuged at 35000 g for 20 min at 4 °C to obtain the outer mitochondria membrane fraction (OM), while the sediment was resuspended in distilled water (mitoplast fraction) and freeze-thawed 4 times. After a 25-min centrifugation at 35000 g the supernatant was separated as matrix (M) fraction. The sediment was resuspended in distilled water and washed twice with centrifugation at 35000 g for 15 min to obtain the inner membrane (IM) fraction.

C) Measurement of mitochondrial methane formation. 2 ml of mitochondrium suspension (0.25 to 5 mg/ml protein) was incubated in 5-ml gas-tight Supelco vials at 37°C in a reaction medium containing 20 mM potassium phosphate buffer (154 mM KCl, 3 mM MgCl_2 , 5 mM succinic acid), 0.1 mM FeCl_3 , 0.06 mM EDTA and 5 mM ASC. The reaction mixture was adjusted to pH 7.4. A 20-min exposure to nitrogen gas was used to induce hypoxia and the reaction was started by the addition of 10 mM H_2O_2 . The samples were kept in a water bath at 37°C for 60 min, the reaction was stopped by immersion in ice-cold water and the samples were subsequently kept on ice until analysis. 200-ml aliquots of the headspace gas were obtained with a Hamilton gas-tight syringe and the rate of methane formation was determined by gas chromatography.

D) Measurement of methane formation in mitochondria subfractions after free radical generation. These experiments were performed in 5-ml gas-tight Supelco vials under continuous stirring. The reaction mixtures contained 2 ml of mitochondrial

Fig. 1. In vitro methane-producing capacity of choline and choline metabolites, using a chemical model of hydroxyl radical generation. See the Methods section for a description of the experiments. The bars indicate mean $\pm$ SD.

fraction (1.5 mg protein/vial) in 20 mM potassium phosphate buffer, 5 mM ASC, 0.1 mM Fe^{3+} /0.06 mM EDTA and 10 mM H_2O_2 (Udenfriend components) in a total volume of 2.5 ml. The reaction was identical to described above, 200-ml aliquots of the headspace gas were obtained and methane formation was determined by gas chromatography.

Tissue culture experiments

The cells were isolated and cultured according to a modified method of Gryglewski *et al.* [9], and Rosen *et al.* [10]. Endothelial cells were verified by light microscopy via the non-overlapping cobblestone morphology and the positive staining for the presence of Factor VIII-related antigen by routine indirect immuno-fluorescence assay [11]. The experiments were performed on 10-day-old endothelial cells ($18\text{--}20 \times 10^6$ cells/flask). Before the start of the studies, the culture medium was changed for Dulbecco's phosphate-buffered saline with or without glucose (5 ml/flask) and the Nunclon caps were changed for Supelco gas-tight caps.

Chemical hypoxia of endothelial cells

Sodium fluoride (NaF) is an inhibitor of enolase and pyruvate kinase and thus inhibits ATP produced by glycolysis. Other inhibitors of anaerobic glycolysis include 2-deoxy-D-glucose (2-DDG), a competitive inhibitor of the uptake and metabolism of glucose, and iodoacetic acid (IAA), which binds to the active site of glyceraldehyde 3-phosphate dehydrogenase and therefore stimulates ATP depletion.

Sodium cyanide (NaCN) and sodium azide (NaN_3) inhibit the oxidation of cytochrome a_3 by molecular oxygen, deactivate mitochondrial complex IV and hence block electron transport, resulting in a decreased level of oxidative metabolism and oxygen utilization.

The uncoupler 2,4-dinitrophenol (2,4-DNP) inhibits mitochondrial oxidative phosphorylation without decreasing oxygen utilization. Further, the hyperglycemia caused by 2,4-DNP induces cellular oxidative stress by increasing superoxide generation.

In these studies, a confluent layer of endothelial cells was exposed to site-specific inhibitors of complex IV of the mitochondrial electron transport chain (NaCN and NaN_3); inhibitors of glycolysis (NaF, 2-DDG and IAA), and the uncoupler of the electron transport from phosphorylation (2,4-DNP) in the concentration range of 1–100 nmol/ml. The oxygen free radical-generating Udenfriend system was used at concentrations of 1–1000 nmol/ml concentration range. Other approaches included the removal of glucose from the reaction medium or exposure to inhibitors of glycolysis, together with metabolic poisons. The total gas volume above the cell layer was 36 ml, and 500- μl gas samples were analyzed in all cases. After measurement of the methane concentration, the contents of the flask were dissolved in 1 M NaOH, and the total protein content was determined by the method of Lowry *et al.*

Granulocyte studies

PMNs were separated from canine venous blood by the method of Guarnieri *et al.* [12]. Following dextran sedimentation and hypotonic lysis (to remove contaminating erythrocytes), Ficoll-Hipaque gradient centrifugation was performed. Cell viability was tested by the trypan blue exclusion method. The PMNs (1.5×10^6 cells/ml) were incubated at 37°C in the presence of 40 μM ferricytochrome c in phosphate buffer, and the generation of superoxide anion was detected with a UV-1601 spectrophotometer (Shimadzu, Japan) at 550 nm. The spontaneous radical production and the phorbol 12-myristate 13-acetate (PMA)-stimulated activity of the PMNs were determined. The extent of release of superoxide into the reaction mixture was calculated from the linear portion of the cytochrome c reduction plot, using a molar absorption coefficient of $19.1 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

Methane measurement

The rate of methane formation was determined by gas chromatography with flame-ionization detection (Carlo Erba Instruments HRGC 5300 Megaserie) and a Chrompack capillary column. The samples were measured in triplicate; the coefficient of variation of the measurements was less than 7%. Possible atmospheric contamination of the samples was investigated; the baseline methane (room air) concentration was determined in separate measurements. For calibration, a standard methane gas mixture was used with a dilution series and fitting a linear regression curve. The methane contents of the experimental samples were determined after the background methane concentration had been subtracted.

Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Differences between groups were evaluated by means of one-way ANOVA tests followed by the Bonferroni t-test. Concentration-dependent within group and between

Fig. 2. Changes in chemiluminescence detected in the presence of 1 mM test compounds (choline metabolites) after induction of the Udenfriend reaction (Figure 2A, upper panel). The degree of inhibition of chemiluminescence was measured with increasing choline (CC), DMSO, mannitol or DMTU concentrations (empty bars: 1 mM, grey bars: 10 mM, black bars: 100 mM) in identical chemical reactions (Figure 2B, lower panel). The bars indicate mean $\pm$ SD.

groups differences were analyzed by two-way ANOVA test. All pairwise multiple comparison procedures were assessed by Bonferroni t-test. Data are expressed as means $\pm$ standard deviation (SD); p values < 0.05 were considered significant.

Results

In vitro studies without biological structures

In this series the methane-generating capacity of PC metabolites was tested in a hydroxyl radical-generating chemical reaction (Figure 1). Approximately 120 000 nmol methane was generated from DMSO. When CC and the choline metabolites were tested, the order of magnitude was lower, but significant methane liberation was detected in the gas phase. For CC, DMEA, MEA and EA, the amount increased linearly with the number of methyl groups in the molecules; 3750 nmol methane was

Fig. 3. Methane production of rat liver mitochondria and mitochondrial subfractions. The results were obtained with intact mitochondria or matrix (M), intermembrane space (IMS), inner membrane (IM) and outer membrane (OM) fractions in a reaction mixture containing K-phosphate buffer, ascorbate and FeCl_3 . The reaction was started by adding hydrogen peroxide and the gas production was measured 60 min after initiation of the reaction. The bars indicate mean $\pm$ SD.

generated from CC, >2960 nmol from DMEA, and >1820 nmol from MEA ($p < 0.05$), whereas for the other test compounds (i.e. EA, betaine, DMG, sarcosine and glycine) significant methane liberation was not observed (Figure 1).

Secondly the lucigenin-enhanced chemiluminescence assay was used to determine the reactive oxygen species-scavenging capacity of the choline metabolites; the findings are depicted in Figure 2A and 2B. In these experiments, the extent of hydroxyl free radical generation in the Udenfriend reaction was quantified by means of the luminol-enhanced chemiluminescence assay in the presence or absence of test compounds, while DMSO, dimethylthiourea (DMTU) and mannitol were used as positive controls.

The methane-producing choline metabolites (i.e. CC, DMEA and MEA) and EA effectively inhibited ROS generation ($p < 0.05$), while the non-methane-producers betaine, DMG, sarcosine and glycine did not display free radical-scavenging capacity (Figure 2A).

CC in the range 1-100 mM dose-dependently decreased the hydroxyl radical concentration generated by the Udenfriend reaction, and exhibited a similar effectiveness as the standard hydroxyl free radical scavengers DMSO, mannitol and DMTU (Figure 2B).

Fig. 4. Effects of chemical hypoxia alone or hypoxia with glucose deprivation on methane generation of control (C) or treated primary aortic endothelial cells. The treatments included glucose deprivation (-Glucose), inhibition of glucose uptake and anaerobic glycolysis using 2-deoxy-D-glucose (2-DDG), iodoacetate (IAA) or sodium fluoride (F) (grey bars indicate 10 μ M, black bars 100 μ M, hatched bars 1 mM).

Experiments with mitochondria

In this system, mitochondria were incubated in gas-tight vials with hydroxyl free radical-generating Udenfriend components (H_2O_2 , ASC and catalytically active iron). For intact mitochondria the results were identical to those reported in our previous paper [4]. As concerns the mitochondrial subfractions, the highest methane production was observed in the matrix (2.96 nmol/mg protein, $p < 0.05$), much less methane was generated in the intermembrane space (0.197 nmol/mg protein), and only very low amounts were detected in the inner membrane (0.08 nmol/mg protein) and outer membrane (0.042 nmol/mg protein) fractions (Figure 3).

Endothelial cell culture studies

The level of methane production increased significantly ($p < 0.05$) over the 2 nmol/mg baseline, reaching ~ 8-10 nmol/mg when intact endothelial cells were deprived of glucose (when glucose was omitted from the reaction mixture) and after the inhibition of glycolysis with NaF, 2-DDG and IAA (Figure 4).

Metabolic inhibition of complex IV by NaCN and NaN_3 increased the methane production further ($p < 0.05$) and resulted in ~ 15 nmol/mg. Simultaneous glucose deprivation or glycolysis inhibition did not enhance this response further (Figure 5).

Higher amounts of liberated methane (20-23 nmol/mg) were observed after the inhibition of oxidative phosphorylation with 2,4-DNP (Figure 6), and similarly high and dose-dependent methane generation was

Fig. 5. Effects of site-specific inhibitors of the mitochondrial electron transport chain (inhibitors of complex IV) sodium cyanide (NaCN) and sodium azide (NaN_3) alone or in combination with glycolysis inhibitors on methane generation of primary aortic endothelial cells (grey bars indicate 1 μ M, black bars 10 μ M, hatched bars 100 μ M). Control (C) = mitochondria without inhibitors.

Fig. 6. Methane generation of control or treated primary aortic endothelial cells. Application of the uncoupling agent 2,4-dinitrophenol (2,4-DNP) in 1-100 μ M range (grey bars indicate 1 μ M, black bars 10 μ M, hatched bars 100 μ M).

measured after the free radical attack of the Udenfriend reaction (Figure 7).

Granulocytes

The PMA-activated PMN granulocyte assay was used to investigate the effects of CC and its metabolites on leukocyte activation. In this system, reactive oxygen species (ROS) were generated upon activation of the NADPH oxidase complex of the PMNs. The methane-producing PC metabolites (i.e. CC, DMEA and MEA) and EA proved to be efficient ($p < 0.05$), while the non-producers, betaine, DMG, sarcosine and glycine did not inhibit the activity of isolated PMN granulocytes (Figure 8).

Fig. 7. Treatment of the cells with increasing concentrations (1-100 μ M) of the hydroxyl radical-generating Udenfriend system. The bars indicate mean $\pm$ SD.

Discussion

In the present study, we carried out a sequential exploration of the possibility of methane generation in aerobic systems under oxido-reductive stress conditions. In the experiments reported here, we proved the possibility of methane generation in a hitherto unrecognized reaction. The results also indicated that methane-generating biomolecules in this pathway may actually counteract ROI generation and exhibit a strong inhibitory potential for the production of hydroxyl radicals. The efficacy of CC was proved to be of the same order as that of the established hydroxyl radical scavenger molecules, mannitol, DMSO and DMTU, and PC metabolites with alcoholic moiety in the molecule displayed an effectiveness proportional to the amount of methane generated and the number of methyl groups in the compounds (see Figures 1, 2 and 9). A similar series of experiments using activated PMN leukocytes furnished evidence that methane-producing PC metabolites inhibit the formation of ROI and may therefore be defined as 'antioxidants'.

The next protocol was designed to determine the possible site of the hypoxic methane generation process in a biological system. The mitochondria are a major source of intracellular ROI production and all components of our chemical model reaction (other than free iron) are ubiquitous constituents in comparatively high concentrations in the matrix [5, 13]. Here, we have demonstrated measurable amounts of mitochondrial methane and that methane generation occurs largely in the matrix. As mitochondria must import most of the vital substances (including choline) required for their proper

Fig. 8. The reduction of cytochrome c by PMA-activated leucocytes (PMNs). PMA-activated PMNs were treated with 1 mM chlorine chloride (CC) or the choline metabolites N-methylethanolamine (MEA), N,N-dimethylethanolamine (DMEA), ethanolamine (EA), betaine, dimethylglycine (DMG), sarcosine and glycine. Each column represents the mean of 3 to 5 determinations with PMNs isolated from the peripheral blood of healthy dogs. The bars indicate mean $\pm$ SD.

Fig. 9. Schematic diagram of the proposed mechanism for the oxido-reductive stress-induced in vivo methanogenic response.

functioning, this suggests biological activity in a location occupied only transiently by PC or choline, rather than a location (i.e. biomembranes) to which they are targeted.

In a further step, we examined the consequences of modulation of the mitochondrial electron transport in cell cultures. In mitochondrial oxidative phosphorylation, substrate oxidation by the electron transport chain creates

a proton gradient across the inner membrane and fuels ATP synthesis by F_0F_1 -ATP synthase. The electron transport chain through which the electrons shuttle must be in a steady state as regards the input of electrons and the availability of the electron acceptor oxygen. A continuing lack of oxygen will cause an abnormally elevated mitochondrial NADH/NAD⁺ ratio and the collapse of ionic homeostasis, leading to dissipation of the transmembrane potential. However, ROI are also produced at significant rates by reactions intrinsic to the normal aerobic metabolism, thus the “redox homeostasis” appropriately includes an intracellular balance between ROI generation and scavenging [14]. A number of conditions lead to impairment of the redox homeostasis, thereby inducing the formation of ROI by transferring the excess electrons to oxygen outside the transport chain, to a site where PC or free choline is present. It must be emphasized that choline may be liberated from membrane-bound PC through the redox-sensitive activation of phospholipase D (PLD). PLD hydrolyzes PC to phosphatidic acid and choline, and it has been implicated as a signal-activated key enzyme in a wide range of physiological responses [15]. In this respect, the redox regulation of PLD is well defined, and it has additionally been proposed that oxidants per se have significant effects on PLD activation in a variety of tissues [16, 17].

In our next experiments, the effects of chemical hypoxia were studied in control or treated primary aortic endothelial cells. For these studies, confluent cultures of porcine aortic endothelial cells were used, the treatments included inhibition of glucose uptake and anaerobic glycolysis, application of site-specific inhibitors of the mitochondrial electron transport chain alone or in combination with glycolysis inhibitors, application of an uncoupling agent, and treatment of the cells with increasing concentrations of the hydroxyl radical-generating Udenfriend system. Methane generation was always observed when compounds with such different modes of primary action were used. The results revealed that disturbance of the normal mitochondrial function leads to significant, dose-dependent methane generation in endothelial cells, the extent depending on the type and intensity of the metabolic distress.

Conclusions

In the 1980s, a series of important studies demonstrated that the formation of reactive oxygen intermediates (ROI) could be a key factor in the acute

cellular responses to hypoxia. It was concluded that superoxide, generated by the one-electron reduction of oxygen, and the formation of hydroxyl radicals through the Fenton or iron-catalyzed Haber-Weiss reaction, play key roles as effectors of the cellular dysfunction and tissue injury that characterize ‘oxidative stress’. Later, it was recognized that, similarly to a dose-response effect, ROI elicit a wide spectrum of cellular responses, ranging from signaling to apoptotic or necrotic cell death [2, 18, 19]. Understandably, research on endogenous antioxidants that control the system has mainly intensified in consequence of *in vitro* radical-scavenging properties of many such compounds.

The formation of methane has previously been thought to be an exclusive attribute of bacteria, producing methane from decomposing organic matter in the gastrointestinal tract of mammals [20, 21]. Nevertheless, a recent study has demonstrated that methane can be generated by plants autonomously, without a protein to catalyze the reaction [22]. The mechanism of plant-derived methane generation is unknown and the findings are currently a subject of debate [23]. Our experiments permit the straightforward conclusion that, in aerobic cells, PC metabolites with a hydroxy moiety produce methane in the mitochondrial matrix. Choline and its metabolites can be oxidized to betaine, DMG, MG and glycine by donating electrons to an electron acceptor (Figure 9). A plausible explanation for the finding that these metabolites generate methane by accepting an electron is that these molecules react according to the changes in the redox milieu, i.e. in accordance with an abnormal shift toward reducing or oxidizing conditions in the cell. The dual functionality of these metabolites has a potential to act as endogenous redox controller of the aerobic organism.

Methane is measurable in the breath of approximately one-third of humans [24]. Although there have been several attempts to link the presence of methane to pathological disorders, the diagnostic value of the breath methane excretion test is still unclear [25-27]. The intra- and intersubject variabilities are usually very large, and the interpretation of the results is often difficult. Methane producers may stop excreting, non-producers may start to excrete methane, and occasionally the methane-producing status does not change after antibiotic treatments targeting the intestinal methanogenic flora [28-30]. Taken together, this suggests also that other, non-bacterial factors are also involved in the mechanism of gastrointestinal methane generation.

It is clear that there are many open questions, and additional investigations are required to prove that

methane formation is a fingerprint of pathologies. Nevertheless, it seems reasonable to assume that the generation of methane may be linked to a cellular response to hypoxia and associated with impaired mitochondrial energy conversion or abnormal ROI generation.

Abbreviations

Reactive oxygen intermediate (ROI); electrophilic methyl group (EMG); phosphatidylcholine (PC); choline chloride (CC); N,N-dimethylethanolamine (DMEA);

N-methylethanolamine (MEA); ethanolamine (EA); dimethylglycine (DMG).

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