Special Issue

Enhancing effects of food components on the production of interferon β from animal cells suppressed by stress hormones*

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Abstract

In today's 'modern' society, no one can escape from the stresses of daily life. Stress stimulates the secretion of stress hormones (e.g. cortisol or noradrenaline) which generally suppress the immune response system, thus rendering the body vulnerable to infectious diseases and cancer. Therefore finding anti-stress food components, which diminish and/or inhibit the stress related suppression of the immune response system would be helpful in maintaining and promoting the health of the human population. Here we established a screening system for anti-stress substances using the cultured human cell line MG-63. The production of interferon- β (IFN- β) by MG-63 cells super-induced by Poly (I): Poly (C) was shown to decrease in a dose dependent manner upon the addition of 0.01–10 $\mu\text{g/ml}$ of cortisol or noradrenaline (NA). 1,2-Diacylglycerol (DG) was demonstrated to abrogate this suppression. Lipid from the fermented milk, kefir, also inhibited the influence of cortisol. Kefiran, a polysaccharide secreted from *L. kaffiranofasiens* GKL-28 diminished the cortisol or NA influenced IFN- β production. But phosphatidylcholine had no significant effect in this system. These results suggest that DG, lipids from kefir and kefiran may be equated as anti-stress food component.

Abbreviations: DG – diacylglycerol; IFN- β – interferon- β ; NA – noradrenaline; PC – phosphatidylcholine; Poly (I):Poly (C) – polyinosinic polycytidylic acid

Introduction

Stress is a reaction which leads to physiological and psychological distortion. For example, atrophy of the thymus and lymph organs, peptic ulcers and hypertrophy of glandula suprarenalis are observed in animals under stress. Stimuli named 'stressor' such as heat, cold, noise, overwork, infection, anxiety and despair are common causes of stress. Once the body is exposed to such stressor, a bioresponse signal is transmitted through limbic system to the cortex glandulae suprarenalis, sympathetic nervous system and medulla glandulae suprarenalis, resulting in the secre-

tion of a large amounts of stress hormones, i.e. cortisol, adrenaline and noradrenaline (NA), into the blood. General responses of the immune system can be suppressed by these hormones (Robert *et al.*, 1991), thus rendering the body vulnerable to infectious diseases and cancer. For example, Cohen *et al.* (1991) reported that there is a positive correlation between psychological stress and susceptibility to respiratory viruses in humans. Ben-Eliyahu *et al.* (1991) demonstrated that stress induces an increase in pulmonary metastases and a decrease in NK cell activity in rats injected with mammary tumor cells. When studying the mechanisms of stress-induced immunomodulation, Dobbs *et al.* (1993) showed that treating mice with a glucocorticoid receptor antagonist reversed the stress-induced suppression of the lymph node cellu-

* This paper is dedicated to the memory of the late professor Hiroki Murakami.

larity in response to local herpes simplex virus infection. When the mice were treated both β -adrenergic antagonist and glucocorticoid receptor antagonist, the stress-induced suppression of the herpes simplex virus-specific CTL activation was reversed. Hermann *et al.* (1995) demonstrated that the glucocorticoid receptor antagonist restored the cellularity to the lymph nodes and enhanced the accumulation of mononuclear cell in lungs of stressed, influenza virus-infected mice.

Therefore finding and utilizing anti-stress food component that can protect the immune response system from the negative effects of stress hormones would be useful in promoting and maintaining human health. Today, however, it is difficult to perform animal experiments, especially stress experiments where inflicting stress animals may be considered inhuman. So *in vitro* tests with cultured animal cells are advisable for the screening of anti stress food components.

Interferons (IFNs) are non-specific humoral factors in the mammalian biodefense system and are produced by most eucaryotic cells in response to a variety of stimulants (virus, endotoxin, chemical inducer Poly (I):Poly (C), etc.) (Stewart, 1980; Pestka S *et al.*, 1987). Though IFN itself has no direct viral killing activity, it initiates intracellular production of several kinds of antiviral proteins which indirectly induces a virus-resistant state. However, stress-related glucocorticoid inhibit synthesis and secretion of interferon by decreasing the production of its mRNA (Gessani *et al.*, 1988). Therefore finding and utilizing anti-stress food component which can prevent the stress-induced suppression of IFN production may allow the body to resist virus infection and proliferation. Osada *et al.* (1994) described the screening of food components which enhance IFN- β production using a cultured cell line and found that sphingomyelin from fermented milk and sphingosine have the ability to greatly enhance the production of IFN- β . Administration of sphingosine also showed remarkable anti-viral activity in mice (Osada *et al.*, in preparation).

We established a screening system for anti-stress food components using cultured human cell lines which have the ability to secrete IFNs in response to stimulants. Kefir is a fermented milk and Kefiran is a polysaccharide derived from kefir. Here we found that 1,2-diacylglycerol (DG), a lipid from Kefir, and Kefiran can inhibit the suppression of IFN production.

Materials and methods

Reagents

DG and phosphatidylcholine (PC), both derived from egg were purchased from Funakoshi Co. Ltd., Tokyo, Japan. Both reagents were dissolved in 10% ethanol/distilled water. Actinomycin D, Poly (I):Poly (C) and NA were obtained from Sigma Chemical Co., St. Louis, Mo. Cycloheximide and cortisol were purchased from Wako Pure Chemicals Industries, Ltd., Osaka, Japan. Poly (I):Poly (C) and cycloheximide were dissolved in phosphate buffered saline (pH 7.0), and actinomycin D was solubilized in ethanol at a concentration of 1 mg/ml. Cortisol was dissolved in 100% ethanol, and NA was solubilized in 0.05 N HCl.

Preparation of lipid from Kefir and Kefiran

Fermented milk, Kefir, was obtained from Nihon Kefir Co. Ltd., Kanagawa, Japan. Total lipids were extracted from Kefir by a method described by Bligh-Dyer (Bligh *et al.*, 1959). Extracted lipids were dissolved in 100% ethanol and diluted in distilled water to 10% ethanol before addition to the screening system.

Kefiran is a polysaccharide secreted by *L. kefir-nofasiens* GKL-28 isolated from Kefir grain. *L. kefir-nofasiens* GKL-28 was cultured in WYAP medium (pH 5.5) for 72 hr at 30. GKL-28 was spun at 8000 rpm for 20 min and the supernatant was transferred to a new tube. Kefiran was obtained by precipitation in 60% ethanol and centrifugation at 8500 rpm for 20 min. The precipitate was dissolved in minimum amount of heated distilled water and centrifuged again at 8500 rpm for 20 min to obtain a water-soluble fraction. This fraction was precipitated in 60% ethanol and centrifuged at 8500 rpm for 20 min. The resulting precipitate was again dissolved in minimum amount of hot distilled water and centrifuged at 8500 rpm for 20 min to obtain a water-soluble fraction. These processes were repeated three times and then the water-soluble fraction was lyophilized. For use during screening, kefir was dissolved in distilled water and autoclaved.

Cells and cell culture

The human osteosarcoma cell line, MG-63 was obtained from American Type Culture Collection (ATCC CRL 1427). MG-63 cells were cultured in Eagle's minimum essential medium (MEM) (Nissui

Figure 1. Protocol for assessing the effects of stress hormone and food components by assaying for the production of IFN- β by MG-63 Cells.

Pharmaceutical Co. Ltd., Tokyo Japan) supplemented with 5% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO₂ at 37 °C.

Assay for detecting the enhancement of IFN- β secretion in the presence of stress hormones

Induction of IFN- β was performed using a method described by Tan *et al.* (1970). As shown in Figure 1, MG-63 (6.5×10^4 cells) in 500 μ l of 5% FBS-MEM medium were plated in each well of a 24 well plate and cultured at 37 °C in an atmosphere of 5% CO₂. After 48 hr the medium was changed to 2% FBS-MEM supplemented with Poly (I):Poly (C), cycloheximide (5 μ g/ml), stress hormone and food components. After culturing for 4 hr, actinomycin D (4 μ g/ml) was added to the medium and further cultured for 1 hr. After washing, the cells were cultured in fresh 2% FBS-MEM for 18 hr. The IFN- β secreted by the cells into medium was measured by using an enzyme-linked immunosorbent assay (ELISA) (Osada *et al.*, 1994).

Results

Suppressive effects of stress hormones on the production of IFN- β from MG-63 cells

Figure 2 illustrates how stress may act on the nervous system to suppress the immune response and how we attempt to control those stress related effects with food

Figure 2. Schematic diagram depicting the transmission pathway of stress and how certain foods may control the effects of stress.

components. Stressor activates the sympathetic nervous system which releases adrenaline and NA, and the hypothalamic-pituitary-adrenal axis which releases glucocorticoid (i.e. cortisol) from the adrenal cortex. These released hormones act to suppress immune response.

IFN- β production by MG-63 cells increased in a dose dependent manner upon the addition of Poly (I):Poly (C) at a concentration range of 0.02–0.08 $\mu\text{g/ml}$. IFN- β secreted by cells induced at any concentration with Poly (I):Poly (C) decreased in a dose dependent manner upon the addition of 0.01–10 $\mu\text{g/ml}$ of cortisol or NA. At the 1–10 $\mu\text{g/ml}$ concentration range, the produced level of IFN- β decreased to about half the control value (Figure 3). No cellular morphological change was observed at these concentrations of cortisol or NA.

These results indicated that Poly (I):Poly (C)-induced IFN- β production can be suppressed by stress hormones in a dose dependent manner, suggesting that moderate concentrations of stress hormones is necessary for establishing a screening system for evaluating food components with anti stress activity.

The effect of DG and PC on the production of IFN- β from MG-63 cells suppressed by stress hormones

Production of IFN- β induced with 0.02 $\mu\text{g/ml}$ of Poly (I):Poly (C) was suppressed upon treatment of cortisol and NA to about half the value of the untreated control (283 IU/ml). The inhibitory effects on IFN- β production by cortisol and NA was diminished by the addition of 5 $\mu\text{g/ml}$ of DG. Furthermore, at a DG concentration of 25 $\mu\text{g/ml}$, the inhibitory effect of cortisol and NA was not only completely abrogated, but the level of IFN- β rose to a value higher than those of control cells (Figure 4). No cellular morphological change was observed at these concentrations of DG. The pattern of the anti stress effect of DG showed a similar pattern of negating the suppression effect of cortisol and NA (Figure 4).

On the other hand, egg derived PC exhibited no significant change on the production of IFN- β in the presence of cortisol (Figure 5), suggesting that PC had no significant anti-stress effect against cortisol in this system.

The effect of lipids from Kefir and Kefiran on the production of IFN- β from MG-63 cells suppressed by stress hormones

Lipids from Kefir exhibited high anti-stress activity. The addition of 1.2 $\mu\text{g/ml}$ of lipid completely reversed the cortisol-induced suppression of IFN- β production. Furthermore, addition of lipids in a range of 3.7–11.1 $\mu\text{g/ml}$ elevated the IFN- β production level about five times higher than control cells (Figure 6a). How-

ever, at the concentrations of 33.3–100 $\mu\text{g/ml}$, IFN- β production levels returned to a value seen for cortisol treated cells in the absence of lipids. No cellular morphological change was observed at these lipid concentrations.

Kefiran exhibited low anti-stress activity. The suppression of IFN- β production by cortisol was diminished in dose dependent manner by the addition of Kefiran in a range of 0.7–167 $\mu\text{g/ml}$ (Figure 6b). At 167 $\mu\text{g/ml}$, Kefiran recovered 70% of the production value as compared to control. A similar anti stress activity pattern was observed for NA (data not shown).

Discussion

We demonstrated here that Poly (I):Poly (C)-induced IFN- β production by MG-63 cells can be suppressed by cortisol and NA. These results suggest that the cultured cell line MG-63 may imitate the *in vivo* condition where the immune system is suppressed by stress. We utilized this system for screening food components containing possible anti-stress activity and found that DG, lipids from Kefir and Kefiran can diminish or abrogate the stress induced suppression of IFN- β production.

IFN- β can be induced by synthetic double-stranded RNA (dsRNA), Poly (I):Poly (C) or virus infection through activation of the transcription factor NF- κ B. Once dsRNA moves into the cytoplasm, dsRNA-dependent protein kinase (PKR) is activated, which in turn activates NF- κ B by phosphorylating I κ B. Phosphorylated I κ B is easily degraded (Kumar *et al.*, 1994). Activated NF- κ B then moves into the nucleus and activates transcription of IFN- β by binding to the positive regulatory domain II (Garoufalidis *et al.*, 1994; Thanos *et al.*, 1995). Conversely, glucocorticoids can suppress the immune response by inhibiting NF- κ B activity by inducing the synthesis of I κ B (Scheiman *et al.*, 1995; Auphan *et al.*, 1995).

The mechanism describing how DG, lipids from kefir and kefiran diminish or abrogate the stress induced suppression of IFN- β production remains unclear. Schutze *et al.* (1992) reported possible signaling pathways for NF- κ B activation. In this pathway, TNF triggers the TNF-responsive PC-specific phospholipase C (PC-PLC) by binding exclusively to the 55 kD TNF receptors. Cytoplasmic 1,2-DG, which is produced by PC-PLC, can activate the two enzymes, PKC and acidic sphingomyelinase (SMase). Acidic SMase hydrolyzes sphingomyelin to produce

Figure 3. The effect of stress hormones on IFN- β production. MG-63 cells were cultured in the presence of various concentration of the stress hormones cortisol (A) and NA (B) to examine their effects on the secretion of IFN- β by cells induced with various concentration of Poly (I):Poly (C).

ceramide, which results in the rapid induction of NF- κ B activity. PKC can also induce NF- κ B activation (Schutze *et al.*, 1990). This suggests that the DG tested in our system may move into cytoplasm with Poly (I):Poly (C) and activates PKC and acidic SMase, leading to NF- κ B activation which results in the enhanced production of IFN- β , overcoming the suppressing effect of cortisol. Sphingomyelin in lipids from kefir has strong enhancing activity for the production of IFN- β (Osada *et al.*, 1994). Kefiran is a water-soluble polysaccharide consisting of approximately equal proportions of galactose and glucose residues (Kooiman, 1968). It also exhibits host-mediated anti-tumor activity (Shiomi *et al.*, 1982). The potential effects of DG, lipids from kefir and kefiran for activating the immune system to overcome the suppressive effect of stress hormones is indicated in this paper. Further studies are necessary to reveal the mechanism.

To our knowledge, this paper is the first to report the establishment of a cell culture system for screening anti-stress food components which may protect the body from the suppression of immune responses

caused by the presence of stress hormones. However, this screening system did not take into consideration that digestion and absorption of foods by digestive tract may be suppressed by stress. Acquiring pure test samples is a laborious and expensive task and using living animals is fraught with many problems. Our screening system can surmount those problems. DG, lipid from kefir and kefiran, which was demonstrated to protect against the stress induced suppression of IFN- β production, may be effective for preventing the proliferation of virus promoted by stress. Further studies are necessary to test whether or not these food components are actually effective as anti-stress agents *in vivo*.

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Figure 4. The effect of 1,2-DG on the production of IFN- β that was suppressed by the stress hormones cortisol (A) or NA (B). 1,2-DG was added to medium at the beginnings of induction period. The amount of IFN- β secreted from the non-cortisol, non-1,2-DG added control was determined to have a value of 100 and the following data was plotted relative to that value. Each value is represented as a mean value ($\pm$ standard deviation) of 2 tests.

Figure 6. The effect of lipids derived from kefir (A) and the polysaccharide Kefiran derived from the cultured medium of *L. kefiranofasiens* GKL-28 (B) on the cortisol induced suppression of IFN- β production. The amount of IFN- β secreted from control cells cultured in the absence of cortisol, lipid, or Kefiran was determined to have a value of 100 and the following data was plotted relative to that value. Each value is represented as a mean value ($\pm$ standard deviation) of 3 tests. Statistically significant difference between samples treated with or without cortisol are marked with the asterisk (***) $p < 0.01$. Statistically significant values between tests samples (dense hatched bar) and control (hatched bar) treated only with 0.5 $\mu\text{g/ml}$ of cortisol are marked with asterisks * $p < 0.05$, ** $p < 0.01$.

Figure 5. The effect of phosphatidylcholine on the cortisol suppressed production of IFN- β . The amount of IFN- β secreted from the non-cortisol, non-PC added control was determined to have a value of 100 and the following data was plotted relatively to that value. Each value is represented as a mean value ($\pm$ standard deviation) of 3 tests. Statistically significant difference between samples treated with or without cortisol are marked with an asterisk (** $p < 0.01$).

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