Linoleic Acid Blocks the Inhibitory Effects of Caffeine on Tumour Promotion by 12-*O*-Tetradecanoylphorbol-13-Acetate in Two-Stage Carcinogenesis in Mouse Skin

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Abstract: The present study assessed the *in vivo* effects of fatty acids on inflammation and carcinogenesis in mice. Fifteen fatty acids were examined for their effects on the inhibitory effect of caffeine on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear oedema in mice. Furthermore, linoleic acid was studied for its effects on the inhibitory effect of caffeine on carcinogenesis in mouse skin initiated with 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by TPA. Among fatty acids, linoleic, γ-linolenic and oleic acids most strongly blocked the inhibitory effect of caffeine on TPA-induced ear oedema. Furthermore, linoleic acid also markedly blocked the inhibitory effect of caffeine on the tumour-promoting activity of TPA. This is the first report to suggest that fatty acids, such as linoleic, γ-linolenic and oleic acids, block the anti-inflammatory activity of caffeine on TPA-induced inflammation in mice. Linoleic acid blocked the inhibitory effect of caffeine on tumour promotion by TPA in the two-stage mouse skin carcinogenesis model. These results suggest that intake of linoleic acid requires attention.

Keywords: Caffeine, fatty acid, linoleic acid, tumour promotion, two-stage carcinogenesis.

1. INTRODUCTION

There are various types of fatty acid in the foods we consume on a daily basis. These fatty acids come from vegetables, meats and fish, and are essential for life. However, administration of a high-fat diet has been observed to promote azoxymethane (AOM)-induced intestinal and colon carcinogenesis in rats [1,2]. Caffeine consumption in the form of coffee and tea is also widespread. In previous studies, topical pre-treatment with caffeine was observed to inhibit tumour promotion by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) following initiation with 7,12-dimethylbenz[*a*] anthracene (DMBA) in mice [3,4]. As coffee or tea is generally consumed with or soon after meals, fatty acids and caffeine are often consumed simultaneously. In this study, fifteen fatty acids were tested for their inhibitory effects on TPA-induced inflammatory ear oedema. Furthermore, the effects of combined linoleic acid and caffeine post-treatment on tumour promotion by TPA in two-stage carcinogenesis in mouse skin were compared with caffeine treatment alone.

2. MATERIALS AND METHODS

2.1. Chemicals

Caffeine, *n*-caproic acid, lauric acid, palmitic acid, stearic acid, tiglic acid, undecylenic acid, oleic acid, linoleic acid, α-linolenic acid, γ-linolenic acid, brassidic acid, DMBA and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St Louis, MO). Behenic acid, elaidic acid, eicosapentaenoic acid and docosahexaenoic acid were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TPA was obtained from Chemicals for Cancer Research Inc. (Chicago, IL).

2.2. Ethical Considerations

Experiments were approved by the Committee for Animal Welfare at the School of Pharmacy, Nihon University, Chiba, Japan, prior to execution, and were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of the School of Pharmacy, Nihon University.

2.3. Animals

Female ICR mice (age: 6 weeks) were purchased from Japan SLC, Inc. (Shizuoka, Japan), and were housed in an air-conditioned specific pathogen-free room (24 ± 1 °C; 50 ± 10% relative humidity; frequency of air charges, 11-19 /h; lights on between 08:00 and 20:00 h) four or five mice per cage. Mice were acclimatised for 1 week before experiments began. Food and tap water were freely available.

2.4. Assay of TPA-Induced Inflammation in Mice

Assays were conducted according to the methods of Yasukawa *et al*. [3]. TPA (1 μg/ear) dissolved in acetone (20 μl) was applied to the right ear of ICR mice (age: 7 weeks) using a micropipette. A volume of 10 μl was delivered to both the inner and outer surfaces of

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ISSN: 2223-3806 / E-ISSN: 1927-5951/15

the ear. Fatty acid (1 mg/ear) or vehicle (20 µl), chloroform-methanol (1:1 v/v), was applied topically about 30 min after each TPA treatment. Caffeine (1 mg/ear) or vehicle (20 µl), chloroform-methanol (1:1, v/v), was applied topically about 30 min before each TPA treatment. For thickness determination, a pocket thickness gauge (Mitsutoyo Co., Ltd., Tokyo, Japan) with a range of 0-9 mm, graduated at 0.01-mm intervals and modified such that contact surface area was increased, thus reducing the tension, was applied to the tip of the ear.

Ear thickness was measured before treatment (a). Oedema was measured at 6 h after TPA treatment (b, TPA plus vehicle; b', TPA plus sample). The following values were then calculated:

Oedema A: oedema induced by TPA plus vehicle (b – a)

Oedema B: oedema induced by TPA plus sample (b' – a)

Inhibitory ratio (%) = [(oedem A – oedema B)/oedema A] × 100

Each value used the mean of individual determinations from four mice.

2.5. Two-Stage Carcinogenesis Experiments

The backs of mice (age: 7 weeks) were shaved with electric clippers. Tumour initiation was accomplished by a single topical application of DMBA (50 µg). Promotion with TPA (1 µg), applied twice weekly, was started one week after initiation. Caffeine (1 mg), caffeine (1 mg) and linoleic acid (1 mg), or vehicle, acetone-DMSO (9:1 v/v; 100 µl), was applied topically 30 min after each TPA treatment. DMBA and TPA were dissolved in acetone and were applied to the shaved area in a volume of 100 µl using a micropipette. The back of each mouse was shaved once a week. The number and diameter of skin tumours were measured every other week, and the experiment continued for 20 weeks. Experimental and control groups each consisted of 15 mice. The average numbers of tumours and standard deviation (S.D.) were calculated using the total numbers for 15 mice. All data were compared for the treatment and control groups each week.

2.6. Statistical Analysis

Statistical differences were tested by Student's *t* test, one-way analysis of variance followed by correction with Tukey-Kramer test and Mann-Whitney *U* exact test.

3. RESULTS

3.1. Effects of Fatty Acids on Inhibitory Action of Caffeine against TPA-Induced Inflammation

Caffeine plus fatty acids were tested for their ability to reduce the intensity of TPA-induced ear oedema in mice. As shown in Table **1**, the inhibitory effects of caffeine against TPA-induced inflammation were

Table 1: Effects of Fatty Acids on the Inhibitory Effects of Caffeine against TPA-Induced Inflammatory Ear Oedema in Mice

Compound	I.R.
Caffeine	74 ± 6.4**
n-Caproic acid	13 ± 6.6
Caffeine + *n*-Caproic acid	76 ± 5.2**
Lauric acid	8.3 ± 5.1
Caffeine + Lauric acid	75 ± 4.7**
Palmitic acid	12 ± 7.6
Caffeine + Palmitic acid	72 ± 7.4**
Stearic acid	12 ± 6.9
Caffeine + Stearic acid	75 ± 5.6**
Behenic acid	38 ± 6.9*
Caffeine + Behenic acid	75 ± 7.2**
Tiglic acid	17 ± 6.0
Caffeine + Tiglic acid	74 ± 8.1**
Undecylenic acid	13 ± 8.0
Caffeine + Undecylenic acid	75 ± 5.5**
Elaidic acid	10 ± 4.2
Caffeine + Elaidic acid	73 ± 9.0**
Oleic acid	11 ± 4.6
Caffeine + Oleic acid	51 ± 7.7**,#
Brassidic acid	40 ± 9.3**
Caffeine + Brassidic acid	81 ± 7.7**
Linoleic acid	0.7 ± 2.3
Caffeine + Linoleic acid	21 ± 8.7##
γ-Linolenic acid	13 ± 8.0
Caffeine + γ-Linolenic acid	36 ± 9.6*,##
α-Linolenic acid	22 ± 8.9
Caffeine + α-Linolenic acid	77 ± 3.5**
Eicosapentaenoic acid	39 ± 6.0**
Caffeine + Eicosapentaenoic acid	76 ± 6.7**
Docosahexaenoic acid	44 ± 4.5**
Caffeine + Docosahexaenoic acid	74 ± 5.2**

Note: Fatty acids were applied 30 min after TPA treatment; ear thickness was determined at 6 h after TPA treatment. I.R. = Inhibition ratio at each 1 mg/ear, mean ± S.D. (%). *$p < 0.01$, ** $p < 0.001$ compared with the control group (Student's *t* test). # $P < 0.05$, ## $P < 0.01$ compared with the caffeine treated group (Student's *t* test).

affected by fatty acids, inhibitory ratio calculated at 6 h, and time of maximum oedema. Of the various fatty acids assayed, the unsaturated fatty acids, oleic acid, linoleic acid and γ-linolenic acid, slightly affected the inhibitory effects of caffeine on TPA-induced inflammation in mice. However, saturated (*n*-caproic, lauric, palmitic, stearic, and behenic acids) fatty acids had no effect. The n-3 fatty acids, α-linolenic acid, EPA and DHA slightly inhibited or had no effect on the inhibitory effects of caffeine on TPA-induced inflammatory ear oedema in mice, while the n-6 fatty acids linoleic acid and γ-linolenic acid blocked the inhibitory effects of caffeine. Linoleic acid had no effect on the inflammatory activity induced by TPA (Table **2**).

Table 2: Effects of Linoleic Acid on TPA-Induced Inflammatory Ear Oedema in Mice

Compounds	Ear oedema[1]
TPA (1.0 μg)	0.28 ± 0.015
TPA (1.0 μg) + linoleic acid (1 mg)	0.28 ± 0.021
TPA (0.1 μg)	0.05 ± 0.007
TPA (0.1 μg) + linoleic acid (1 mg)	0.06 ± 0.013

Note: Fatty acids were applied 30 min after TPA treatment; ear thickness was determined at 6 h after TPA treatment. [1]Mean ± S.D. (mm).

3.2. Effects of Linoleic Acid on Inhibitory Effects of Caffeine against TPA-Induced Tumour Promotion

Figure **1A** shows the time course of skin tumour formation in groups of mice treated with DMBA plus TPA, and caffeine with or without linoleic acid. The first tumour appeared at week 5 in the group treated with DMBA plus TPA, and in that treated with DMBA plus TPA and caffeine plus linoleic acid. In the group treated with DMBA plus TPA and caffeine, the first tumour appeared at week 7. The proportion of tumour-bearing mice reached 100% at weeks 9 and 16 in the group treated with DMBA plus TPA, and in that treated with DMBA plus TPA and then caffeine plus linoleic acid, respectively. The proportion of tumour-bearing mice in the group treated with DMBA plus TPA and caffeine was 40% at week 20. The data in Figure **1B** show that caffeine decreased the average number of tumours per mouse. Treatment with DMBA plus TPA without caffeine produced 11.3 tumours per mouse at week 20, whereas treatment with DMBA plus TPA and caffeine produced 5.3 tumours per mouse. Thus, post-treatment with caffeine resulted in a 53% reduction in the average number of tumours per mouse at week 20. In contrast, the addition of linoleic acid to caffeine following DMBA plus TPA treatment blocked inhibitory activity of caffeine on tumour promotion; this group displayed 10.0 tumours per mouse.

Topical post-treatment of caffeine suppressed tumour promotion by TPA in DMBA-initiated mice, but the inhibitory effects of caffeine were blocked by linoleic acid.

4. DISCUSSION

Our previous studies have demonstrated that several natural compounds present in foods and dietary supplements inhibit tumour promotion during

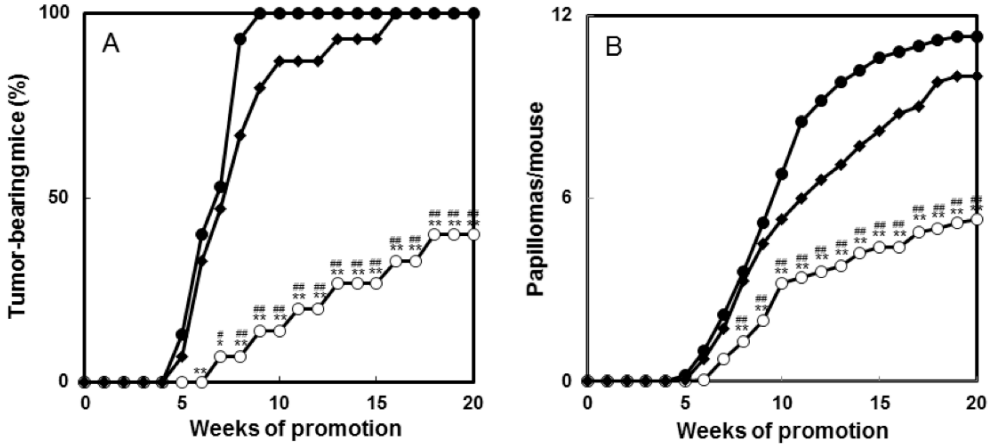

Figure 1: Effects of linoleic acid on the inhibitory effects of caffeine against the promotion of skin papillomas produced by TPA following DMBA initiation in mice.

From 1 week after initiation with a single topical application of 50 μg of DMBA, 1.0 μg of TPA was applied twice weekly. Topical application of caffeine (1 mg/mouse), caffeine and linoleic acid (each 1 mg/mouse), or vehicle was performed 30 min after each TPA treatment. Data are expressed as a percentage of mice bearing papillomas (**A**), and as average numbers of papillomas per mouse (**B**). ● = +TPA with vehicle alone; ○ = +TPA with caffeine; ♦ = +TPA with caffeine and linoleic acid. *$p < 0.05$, **$p < 0.01$ = compared with control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ = compared with caffeine plus linoleic acid-treated group.

two-stage carcinogenesis in mouse skin [3-7]. In addition, we observed that combined treatment with glycyrrhizin and caffeine yielded stronger inhibition than treatment with either of these compounds alone [4]. Pre-treatment with caffeine was observed to inhibit tumour promotion by TPA [3,4]. In this study, post-treatment with caffeine inhibited tumour promotion by TPA in two-stage carcinogenesis in mouse skin, but this inhibitory effect was blocked by linoleic acid.

We found that n-6 fatty acids and mono-unsaturated fatty acids blocked the inhibitory effects of caffeine on TPA-induced inflammatory ear oedema in mice. The inhibitory effects against TPA-induced inflammation have been demonstrated to closely parallel those of the inhibition of tumour promotion in two-stage carcinogenesis initiated by DMBA and TPA, a well-known tumour promoter, in a mouse skin model [8] as n-6 fatty acids block the inhibitory effects of anti-tumour promoters on tumour promotion in two-stage carcinogenesis in mouse skin.

Fatty acids are important components of foods and dietary supplements. Polyunsaturated fats are thought to protect against cardiovascular disease by providing more membrane fluidity than monounsaturated fats, but they are more vulnerable to lipid peroxidation (rancidity). On the other hand, some monounsaturated fatty acids (in the same way as saturated fats) may promote insulin resistance, whereas polyunsaturated fatty acids may be protective against insulin resistance [9,10]. Levels of oleic acid, along with other monounsaturated fatty acids, in red blood cell membranes are positively associated with breast cancer risk. The saturation index of the same membranes was inversely associated with breast cancer risk [11].

High-fat diet promoted AOM-induced intestinal tumours in rats [1]. In addition, unsaturated fats had a greater promoting activity than saturated fat on AOM-induced colon carcinogenesis in rats [2]. In this study, the inhibitory effects of caffeine on TPA-induced inflammation in mice were blocked by linoleic acid, while saturated fatty acids had no effect. Some unsaturated fatty acids, including oleic acid, linoleic acid and γ-linolenic acid, blocked the inhibitory effects of caffeine on the inflammation induced by TPA. In contrast, n-3 fatty acids did not affect the blocking effects of caffeine on inflammation induced by TPA in mice, but n-6 fatty acids blocked the inhibitory effects of caffeine. Furthermore, the inhibitory effects of caffeine on tumour promotion by TPA, after initiation with DMBA in mouse skin, were blocked by linoleic acid, and linoleic acid was shown not to reinforce the effects of TPA. Sakaguchi *et al*. reported that a diet rich in unsaturated fatty acids (5% linoleic acid ethyl ester) promoted AOM-induced colon tumours in rats, but that a diet rich in saturated fatty acids (4.7% stearic acid ethyl ester) had no effect in the same model [2]. Akihisa *et al*. reported that DHA inhibited tumour-promoting activity by TPA in two-stage carcinogenesis in mouse skin [12]. Harvey *et al*. reported that long-chain saturated fatty acids are able to induce pro-inflammatory responses and significantly impact growth and viability of endothelial cells [13]. On the other hand, oleic acid reduces the inflammatory effects of long-chain saturated fatty acids on human aortic endothelial cells by reducing cellular stearic acid incorporation and nuclear factor-κB activation [14]. Among dietary fatty acids, n-3 fatty acids, conjugated linoleic acid isomers and *trans* fatty acids activate an inhibitory G protein-coupled receptor-mediated pathway that specifically suppresses tumour uptake of saturated, monounsaturated and n-6 polysaturated fatty acids, thereby inhibiting early steps in the linoleic acid-dependent growth-promoting pathway [15].

Numerous chronic diseases, including cardiovascular disease, diabetes, cancer, obesity, autoimmune diseases, rheumatoid arthritis, asthma and depression, are associated with increased production of thromboxane A_2 (TXA_2), leukotriene B_4 (LTB_4), IL-1β, IL-6 and TNF. Simopoulos reported that all of these factors increase with n-6 fatty acid intake and decrease with increases in n-3 fatty acid intake [16].

These results suggest that although unsaturated fatty acids are important nutrients, consumption of large quantities is inadvisable.

CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

FINANCIAL DISCLOSURE

Grant Support

This study was supported in part by a General Individual Research Grant, Nihon University.

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Received on 01-06-2015 Accepted on 08-08-2015 Published on 25-08-2015

DOI: http://dx.doi.org/10.6000/1927-5951.2015.05.03.4