Local Thrombus Formation in the Site of Intravenous Injection of Chlorpromazine: Possible Colloid-Osmotic Lysis of the Local Endothelial Cells

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Since amphiphilic drugs are known to interact with biomembranes, we investigated local vessel damage and thrombosis which might be brought about by intravenous dosing using chlorpromazine (CPZ) as a representative compound. CPZ-induced hemolysis was suppressed by an increase in sucrose concentration in the medium, characterizing this hemolysis to be colloid-osmotic lysis, which includes the enhancement of membrane phospholipid fluidity and consequent small pore formation in the membranes. This was supported by the observation that hemolysis by filipin, not featuring the stage of small pore formation, was not affected by sucrose. [^{14}C]Glucose-entrapping liposomes were degraded by CPZ, and this degradation was enhanced by an increase in the intravesicle glucose concentration. These results indicated that the compound could induce colloid-osmotic lysis in erythrocytes and artificial membrane vesicles. CPZ also injured cultured porcine aortic endothelial cells (PAEC), as evidenced by lactate dehydrogenase (LDH) leakage. This injury was also suppressed by increase in sucrose concentration in the medium, suggesting that colloid-osmotic lysis again occurred. When rats were intravenously injected with CPZ, local endothelial cell (EC) injury and associated thrombus formation were observed, suggesting that CPZ's action was also evident *in vivo*. To our knowledge, this is the first finding which suggests that an intravenously dosed amphiphilic drug can injure local ECs based on a colloid-osmotic lysis mechanism leading to thrombosis.

Key words chlorpromazine; endothelial cell; thrombosis; amphiphile; colloid-osmotic lysis

When a blood vessel suffers injury followed by hemorrhage, the hemostatic system operates to limit the damage and protect the organism. This involves platelet adhesion to the injured site through recognition of the von Willebrand factor by specific receptors, followed by platelet aggregation promoted by members of the integrin family, and finally by thrombus formation at the damaged site.¹⁾

However, if an undesirable thrombus forms as a result of endothelial damage caused by some foreign substances, it is regarded as a toxic reaction.²⁾ This is the case, for example, with phosphodiesterase III inhibitors which induce arterial lesions by disrupting vascular physiology,³⁾ and with some toxic plant lectins which induce endothelial cell (EC) death by their own cytotoxicity.⁴⁾ However, these reactions might be predictable to some extent by taking their modes of action into consideration.

There are numerous low molecular weight drugs with various pharmacological activities, not necessarily related to endothelial biochemistry, which are directly injected into the vasculature. Since drug molecules are generally designed to possess membrane permeability to facilitate delivery to their target tissues, drugs used in intravenous formulations may often be amphiphilic. Such compounds are known to interact with cell membranes and possibly cause cell damage by mechanisms not associated with their pharmacological activities.^{5–7)} If such a drug damages ECs, it might result in thrombosis, and in fact, a few instances have been reported.^{8,9)} However, the mechanisms underlying this kind of thrombosis have received little attention so far, possibly because of the lack of any obvious close relation to endothelium physiology. Only pathological assessment using laboratory animals can provide perspectives for such toxicity.²⁾ Therefore, we consider it important, from the viewpoint of

pharmaceutical development, to investigate amphiphile-induced thrombosis after intravenous dosing, and to clarify the causal factors.

We chose chlorpromazine (CPZ), a dopamine and norepinephrine antagonist being used as a neuroleptic, as a model compound. Though CPZ is an oral drug, we considered it a suitable model for the present investigation because CPZ is a well-known amphiphilic drug with cell lytic activity which has been investigated extensively. The mechanism of cell lysis, principally hemolysis, by CPZ is accepted as so-called "colloid-osmotic lysis" initiated by interaction with cell membranes based on its amphiphilicity.^{10,11)} In the present study, we (1) investigated whether CPZ-induced colloid-osmotic lysis can take place not only in erythrocytes but also in other cells such as vascular ECs, and (2) examined the potential of CPZ to induce thrombosis associated with local EC damage after intravenous administration.

MATERIALS AND METHODS

Chemicals CPZ hydrochloride, dicetyl phosphate, cholesterol and Triton X-100 were purchased from Nacalai Tesque (Kyoto, Japan), Tween 20 from Wako Pure Chemical Industries (Osaka, Japan), filipin (as filipin III) and L- α -phosphatidylcholine from Sigma (St. Louis, MO, U.S.A.), and D-[U- ^{14}C]glucose from Amersham Life Science (Buckinghamshire, UK). All other chemicals were of reagent grade.

Hemolysis A blood sample was taken from a male Sprague-Dawley rat (10 weeks old, Charles River Japan, Kanagawa, Japan), and an erythrocyte suspension was prepared by 15-fold dilution with a 10 mM sodium phosphate buffer solution containing either 150 mM NaCl (buffer A), 10 mM sucrose and 145 mM NaCl (buffer B), or 40 mM sucrose

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and 130 mM NaCl (buffer C). To erythrocyte suspensions (2 ml) prepared in each buffer A, B or C, CPZ (2 mM) or filipin (6 μ M) solution (2 ml) dissolved in the same buffer was added, followed by incubation at 37 °C. After 5, 15 and 30 min, relative hemolysis (%) was determined by measuring the decrease in the light scattering at 740 nm¹²⁾ using a UV-VIS spectrometer (U-3210, Hitachi, Tokyo, Japan) and calculated using the following formula:

$$\text{relative hemolysis (\%)} = (b - a) / b \times 100$$

where *a* and *b* are the absorbance values after incubation (in buffer A, B and C) with and without (buffer control) the test substances, respectively, setting the absorbance value when distilled water (2 ml) was mixed with the erythrocyte suspension (relative hemolysis is 100%) as zero.

Liposome Degradation Assay Preparation of liposomes entrapping [¹⁴C]glucose and their degradation assay were performed according to the methods of Oku *et al.*¹³⁾

1- α -Phosphatidylcholine (2 μ mol) and cholesterol (1 μ mol) were dissolved in chloroform, and dicetyl phosphate (0.2 μ mol) in 50% methanol. The combined lipid solution in a round-bottomed flask was evaporated to dryness. To the formed dry lipid film was added a 200 μ l aqueous solution containing glucose and NaCl (total osmolarity of 300 mosM) and a tracer amount of [¹⁴C]glucose, then the mixture was allowed to swell at 37 °C for 30 min and vortexed vigorously. Untrapped glucose was removed by overnight dialysis against 150 mM aqueous NaCl, and subsequent gel filtration using a PD-10 column (Pharmacia, Sweden) eluted with aqueous 150 mM NaCl as the eluent. The void fractions were combined to obtain the liposome suspension for the degradation assay, with entrapped glucose concentrations of 50, 100, 200 and 300 mM (NaCl concentrations of 125, 100, 50 and 0 mM, respectively).

Liposome suspension (50 μ l) and CPZ solution in 150 mM NaCl (250 μ l) were mixed (a final drug concentration of 2 mM) and left at room temperature. After 10 and 30 min, the reaction mixture was poured onto a membrane filter (GVWP, 220 nm, Milipore, Bedford, MD, U.S.A.), filtered by aspiration, and washed with 2 ml of 150 mM NaCl twice. The filter was placed in a scintillation vial to which were added distilled water and scintillator cocktail (Emulsifier-Scintillator PLUSTM, Packard, Meriden, CT, U.S.A.), and the trapped radioactivity on the filter which represents the intact liposomes was measured with a liquid scintillation counter (2500TR, Packard). Degradation rate (%) was calculated using the following formula:

$$\text{degradation rate (\%)} = (b - a) / (b - c) \times 100$$

where *a*, *b* and *c* are the dpm values after treatment with CPZ, 150 mM NaCl (without the test compound; control) and 0.2% (v/v) Triton X-100 (100% degradation), respectively.

Assay of EC Injury Normal porcine aortic endothelial cells (PAEC) were purchased from Cell Systems (Kirkland, WA, U.S.A.). In order to investigate the effects of sucrose on CPZ-induced cytotoxicity, CPZ was dissolved in buffer D (10 mM Tris-HCl, containing 150 mM NaCl, pH 7.4), buffer E (10 mM Tris-HCl, containing 20 mM sucrose and 140 mM NaCl, pH 7.4) or buffer F (10 mM Tris-HCl, containing 40 mM sucrose and 130 mM NaCl, pH 7.4).

PAEC were cultured in a serum-supplemented medium

(CS-C Medium, Cell Systems) and transferred to 96-well (6.4 mm diameter) tissue culture plates (approximately 5000 cells/well). Cells were incubated overnight at 37 °C, and after confirmation of growth to confluence, the medium was removed. CPZ (1 mM) or control solution in buffer D, E or F (100 μ l) was added to each well, and after incubation at 37 °C for 5, 10 and 30 min, an aliquot of the medium (50 μ l) was sampled and subjected to lactate dehydrogenase (LDH) assay using an assay kit (LDH-Cytotoxic Test Wako, Wako Pure Chemical Industries), with measurement of the absorbance at 550 nm. Relative cytotoxicity (%) was calculated using the following formula:

$$\text{relative cytotoxicity (\%)} = (a - b) / (c - d) \times 100$$

where *a* and *b* are the absorbance values after treatment with CPZ and buffer of the corresponding sucrose concentration without CPZ (control), respectively; *c* is the absorbance value after treatment with 0.5% (v/v) Tween 20 for 1 h (the relative cytotoxicity is 100%); and *d* is the absorbance value when buffer D was directly subjected to the LDH assay (reagent blank).

Animal Study CPZ was dissolved in a vehicle (5% mannitol solution; pH adjusted to 4 with HCl in order to improve the CPZ solubility) and administered to male Sprague-Dawley rats (Charles River Japan) *via* the tail vein at a daily dose of 10 mg (HCl salt)/5 ml/kg/d for 5 d. Control rats received the vehicle at 10 ml/kg/d for 5 d. The rate of injection was 10 ml/min. Animals were sacrificed under ether anesthesia at 4 h after the final treatment and subjected to necropsy. The tails were fixed in neutral-buffered 10% formaldehyde, and tissue samples including the lateral caudal veins were removed from the injection site. The tissues were dehydrated, embedded in paraffin, sectioned at approximately 5 μ m in thickness, stained with hematoxylin and eosin, then examined under a light microscope.

Statistical Analysis In order to evaluate the effects of sucrose or glucose concentration on the cell or liposome degradation rate, a linear regression analysis was carried out with data at the initial incubation time point, using SAS Version 6.12 (SAS Institute, Inc., Cary, NC, U.S.A.). The acceptable level of significance was set at *p* < 0.05.

RESULTS

Hemolysis One of the convincing methods to demonstrate that hemolysis is due to colloid-osmotic lysis is to determine the suppressive effects of colloid species (*e.g.*, sucrose, glucose and dextran) on the cell lysis rate, as has been performed by Morimoto *et al.*¹¹⁾, Kastu *et al.*¹⁴⁾ and Harris *et al.*¹⁵⁾ As shown in Fig. 1, the rate of CPZ-induced hemolysis appeared to be suppressed in accordance with the increase in sucrose concentration in the medium. On the other hand, hemolysis induced by filipin, a polyene antibiotic, was not affected by the sucrose concentration. In the control experiments without CPZ or filipin, hemolysis occurred only slightly and the rate was not affected by the sucrose concentration (data not shown). Statistical evaluation was based upon the initial phase of the reaction, because the difference in the relative hemolysis becomes naturally smaller as the reaction progresses¹⁵⁾. Thus, a linear regression was carried out between the sucrose concentration and initial hemolysis rate

Fig. 1. Effects of Sucrose on Hemolysis Induced by Chlorpromazine (CPZ) and Filipin

Final concentrations of CPZ and filipin were 1 mM and 3 μ M, respectively. Sucrose concentrations in the buffer solutions were 0 mM ($\blacktriangle$), 10 mM ($\blacksquare$) and 40 mM ($\bullet$). Each point represents the mean of data from 2 experiments (the relative range of values between the 2 assays, i.e., difference divided by mean value, was less than 6%).

(i.e., the data at 5 min), resulting in a significant correlation for CPZ with a negative value for the slope, and a non-significant correlation for filipin. These data indicate that the mechanism of hemolysis by CPZ but not filipin is colloid-osmotic lysis.

Liposome Degradation Liposomes entrapping various concentrations of glucose were prepared, and the degradation by CPZ was determined in terms of [14 C]glucose release (Fig. 2). In this case, colloid-osmotic lysis of the liposome is enhanced as the concentration of the colloid component (glucose) is elevated, because the colloid exists in the intravesicle space, as described by Oku *et al.*¹³⁾

In the control experiment without CPZ, all liposome preparations were stable (*ca.* 100% intact) during incubation (data not shown). Therefore, CPZ could cause the degradation of liposomes, and the degradation was facilitated by an increase in the intravesicle glucose concentration, while the total intravesicle osmolarity in all preparations was the same (300 mosM). Statistical analysis was carried out based on the same concept as in the case of hemolysis. Although there was variance within each liposome preparation, the correlation between the glucose concentration and the degradation at 10 min was statistically significant, with a positive value for the slope. Thus CPZ caused colloid-osmotic lysis even in artificially-prepared membrane vesicles.

PAEC Cytotoxicity Cytotoxicity of CPZ to PAEC was assessed by LDH leakage (Fig. 3). Even in the control experiment without CPZ, LDH leakage was observed to some extent (*ca.* 10% at 30 min), but this baseline cytotoxicity was

Fig. 2. Effects of Intravesicle Sucrose Concentration on 14 C Release Induced by Chlorpromazine (CPZ) from Liposomes Entrapping Various Concentrations of [14 C]Glucose

The final concentration of CPZ was 2 mM. Intravesicle glucose concentrations were 50 mM ($\bullet$), 100 mM ($\blacksquare$), 200 mM ($\blacklozenge$) and 300 mM ($\blacktriangle$). Each point represents the mean $\pm$ S.D. of 3 liposome preparations.

Fig. 3. Effects of Sucrose on LDH Leakage from PAEC Induced by Chlorpromazine (CPZ)

The final concentration of CPZ was 1 mM. Sucrose concentrations in the buffer solutions were 0 mM ($\blacktriangle$), 10 mM ($\blacksquare$) and 40 mM ($\bullet$). Each point represents the mean $\pm$ S.D. of 3 experiments.

not affected by the sucrose concentration (data not shown). CPZ was observed to induce LDH leakage from PAEC, and this leakage was suppressed as the sucrose concentration in the medium was elevated. A significant correlation between the sucrose concentration and degradation at 5 min was observed with a negative value for the slope. These data suggest that CPZ can also cause colloid-osmotic lysis in ECs.

In Vivo Study The effects of intravenous dosing of CPZ on the local caudal vein were investigated in rats. On necropsy, thrombi extending over 2 cm downstream from the injection sites were observed in all CPZ-treated rats (3 animals), while there were no abnormal findings in the control rats (3 animals). Macroscopically, no thrombi were apparent in the vasculature except at the injection sites for both groups. Typical photomicrographs of lateral caudal veins at the injection sites are shown in Fig. 4. In the CPZ-treated rat, vascular ECs were injured and lost, and thrombus formation was apparent (Fig. 4A). In control rats, normal vascular ECs were observed, with no remarkable findings in the vessels, except for perivascular edema due to the injection procedure (Fig. 4B).

Fig. 4. Light Micrographs of the Lateral Caudal Veins (Injection Site) of Rats Receiving Repeated Intravenous Dosing of Chlorpromazine (CPZ) or the Vehicle

A($\times 100$), CPZ (10 mg/5 ml/kg/d, for 5 d); B($\times 150$), Vehicle control (10 ml/kg/d for 5 d). (A) Note loss of vascular endothelial cells (arrows) and the thrombus formation. (B) Normal vascular endothelial cells with purple-stained nuclei are apparent (arrows).

DISCUSSION

Since the 1960's, numerous investigations of the interaction of amphiphilic drugs with biomembranes have been carried out.^{6,16,17} Among such amphiphiles, CPZ is a representative compound that has received great attention through studies using erythrocytes. One of the well-known CPZ actions is colloid-osmotic hemolysis (as observed in the present paper; Fig. 1), and this is considered to occur after the formation of "small pores" in the membrane.¹⁰

Another reported phenomenon with CPZ is protection against hypotonic hemolysis (osmotic resistance; OR).⁶ The proposed mechanism of OR involves: intercalation of the CPZ molecule with the membrane, rearrangement of membrane phospholipids accompanied by redistribution of the drug molecule, "small pore" formation in the membrane, an increase in permeability of small electrolytes, and a decrease in the osmotic difference between inner and outer media¹⁸. This mechanism could be supported by the fact that filipin, which does not induce lipid fluidity and small pore formation in its hemolytic process,^{19,20} does not exhibit OR.¹⁶ Concurrently, filipin did not evoke colloid-osmotic hemolysis (Fig. 1). Therefore, the onset of small pore formation in CPZ-induced hemolysis could be explained by the mechanism of OR. In this way, we are able to provide a comprehensive interpretation of CPZ-induced hemolysis: (1) molecular insertion into the membrane, (2) phospholipid rearrangement, (3) small pore formation, (4) influx of small electrolytes, and finally (5) osmotic rupture.

Because of its nature, CPZ-induced colloid-osmotic lysis would be expected to occur with other tissue cell membranes in contact with extracellular fluid. Therefore, we determined CPZ-induced degradation of liposomes, artificial membrane vesicles which contain neither membrane proteins nor intracellular organelle. Indeed, CPZ was able to degrade [¹⁴C]glu-

cose-entrapping liposomes, and the rate increased with the intravesicle concentration of glucose (acting as "colloid") (Fig. 2), indicating that CPZ induces colloid-osmotic lysis of liposomes. This observation leads to an implication that CPZ-induced colloid-osmotic lysis can occur in cell membranes in other tissues.

In view of its physiological significance, great attention must be paid to local vascular damage and subsequent thrombosis after intravenous dosing of amphiphilic drugs. Although tissues located downstream, like the lung, coronary artery, brain and kidney, might also suffer from drug exposure and injury, we focused on local ECs because we can assume significant higher level of drug exposure and risk of injury at this site than at the other tissues where the drug is extensively diluted by blood.

As expected, CPZ was found to induce the damage of cultured ECs as evident from LDH leakage. In addition, leakage of this cytosol marker was suppressed by increases in sucrose concentration in the medium, again suggesting that the mode of action is colloid-osmotic lysis (Fig. 3).

When CPZ was given intravenously to rats, thrombi at the injection site were observed at necropsy. On light microscopic inspection, loss of endothelium due to cell injury and thrombus were observed at the injection site (Fig. 4A). The systemic thrombogenic potential of CPZ has been argued^{21,22} due to a fact of elevated levels of anti-cardiolipin antibodies and lupus anticoagulant in patients with long-term oral CPZ treatment,²¹ since these anti-phospholipid antibodies may confer an increased risk of thrombosis.²³ However, it has been shown through investigations on numbers of patients with chronic CPZ treatment, that CPZ itself does not essentially predispose a patient to thrombosis or related vascular damage associated with these autoantibodies.^{21,23,24} Furthermore, in our study, thrombi were found only at the injection sites, not systemically. Therefore, our data suggest that CPZ

induces colloid-osmotic lysis of local ECs in the injection site *in vivo*, and consequent injury could lead to the local formation of thrombus. Exposure to CPZ in this animal study was somewhat different from that *in vitro*, the concentration in the dosing solution (5.6 mM) being higher than applied *in vitro* (hemolysis and PAEC injury, 1 mM; liposome degradation, 2 mM); also the exposure time during the bolus injection was only approximately 10 s. However, for the *in vitro* studies, CPZ concentrations were practically constrained by the following 2 factors: (1) CPZ concentration should be high enough to induce membrane destruction sufficient for detecting clear effects of colloid components; (2) CPZ has limited solubility in an assay media. For the *in vivo* study, we employed a CPZ concentration at which endothelium injury could be detected with a conventional dosing regimen. Thus, it was difficult to conduct an *in vivo* experiment with the same exposure conditions (concentration of CPZ, concentration of colloid component, exposure time) as applied *in vitro*. However, we think that this is the first finding which suggests that an intravenously dosed amphiphilic drug can injure local ECs based on the colloid-osmotic lysis mechanism leading to thrombosis.

Since ADP leakage from erythrocytes can facilitate platelet aggregation and thrombus growth²⁵, prominent hemolysis at the injection site, caused by a high degree of drug exposure, might partially contribute to thrombus formation.

Finally, taking the mechanism of CPZ-induced cell injury into account, it can naturally be assumed that other amphiphiles would also induce colloid-osmotic cell lysis. In fact, several alcohol derivatives have been reported to produce phospholipid fluidity (flip-flop) in erythrocyte membranes and colloid-osmotic hemolysis²⁶, and aprindine, an amphiphilic intravenous antiarrhythmic drug, evoked thrombosis at the injection site associated with vascular injury in an animal toxicity study⁸. Thus, it could be hypothesized that any amphiphilic drug given intravenously, with enough activity for colloid-osmotic cell lysis, could have the potential to induce vascular damage and thrombosis, as in the case of CPZ.

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REFERENCES

- 1) Ware J., Russel S. R., Vicente V., Scharf R. E., Tomer A., McMillan R., Ruggeri Z. M., *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 2026—2030 (1990).
- 2) Zbinden G., Grimm L., *Arch. Toxicol. Suppl.*, **8**, 131—141 (1985).
- 3) Joseph E. C., Jones H. B., Kerns W. D., *Toxicol. Pathol.*, **24**, 429—435 (1996).
- 4) Hughes J. N., Lindsay C. D., Griffiths G. D., *Human Exp. Toxicol.*, **15**, 443—451 (1996).
- 5) Kodavanti U. P., Mehendale H. M., *Pharmacol. Rev.*, **42**, 327—354 (1990).
- 6) Seeman P., *Pharmacol. Rev.*, **24**, 583—655 (1972).
- 7) Lüllmann H., Lüllmann-Rauch R., Wassermann O., *CRC Crit. Rev. Toxicol.*, **4**, 185—218 (1975).
- 8) Ishimura K., Mitsuzono T., Ogawa T., Isowa K., Kashima H., Hatano M., *Jpn. Pharmacol. Ther.*, **15**, 1023—1049 (1987).
- 9) Mariscal I., Moffat C., Huebner G., *Scand. J. Haemat.*, **10**, 390—340 (1973).
- 10) Lieber M. R., Launge Y., Weinstein R. S., Steck T. L., *J. Biol. Chem.*, **259**, 9225—9234 (1984).
- 11) Morimoto Y., Yutani Y., Kado Y., Iida T., Shiota M., Takeuchi Y., *Biol. Pharm. Bull.*, **18**, 1535—1538 (1995).
- 12) Zhou M.-J., Petty H. R., *J. Cellular Physiol.*, **157**, 555—561 (1993).
- 13) Oku N., Nojima S., Inoue K., *Biochim. Biophys. Acta*, **595**, 277—290 (1980).
- 14) Katsu T., Ninomiya C., Kuroko M., Kobayashi H., Hirota T., Fujita Y., *Biochim. Biophys. Acta*, **939**, 57—63 (1988).
- 15) Harris R. W., Sims P. J., Tweten R. K., *Infect. Immun.*, **59**, 2499—2501 (1991).
- 16) Seeman P., *Biochem. Pharmacol.*, **15**, 1767—1774 (1966).
- 17) Fujii T., Sato T., Tamura A., Wakatsuki A., Kanaho Y., *Biochem. Pharmacol.*, **28**, 613—620 (1979).
- 18) Hägerstrand H., Isomaa B., *Chem.-Biol. Interact.*, **79**, 335—347 (1991).
- 19) Goldstein D. B., *Ann. Rev. Pharmacol. Toxicol.*, **24**, 43—64 (1984).
- 20) Seeman P., *Fed. Proc.*, **33**, 2116—2124 (1974).
- 21) Canoso R. T., de Oliveira R. M., *Am. J. Hematol.*, **27**, 272—275 (1988).
- 22) Ducloux D., Florea A., Fournier V., Rebibou J. M., Chalopin J. M., *Nephrol. Dial. Transplant.*, **14**, 1335—1336 (1999).
- 23) McNeil H. P., Chesterman C. N., Krilis S. A., *Adv. Immunol.*, **49**, 193—280 (1991).
- 24) Metzger W. S., Canoso R. T., Newton J. E., *South Med. J.*, **87**, 190—192 (1994).
- 25) Stormorken H., *Fed. Proc.*, **30**, 1551—1555 (1971).
- 26) Schwichtenhövel C., Dueticke B., Haest C. W. M., *Biochim. Biophys. Acta*, **1111**, 35—44 (1992).