Clomiphene, an Ovulation-Inducing Agent, Causes $[Ca^{2+}]_i$ Increases in Human Osteoblast-Like Cells

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Abstract

The effect of clomiphene, an ovulation-inducing agent, on cytosolic free Ca^{2+} levels ($[Ca^{2+}]_i$) in MG63 human osteosarcoma cells was explored by using fura-2 as a Ca^{2+} indicator. Clomiphene at concentrations between 5-75 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner with an EC_{50} of 50 μM. The $[Ca^{2+}]_i$ signal consisted of an initial rise and a sustained phase. Ca^{2+} removal reduced the Ca^{2+} signal by 40±10%. The $[Ca^{2+}]_i$ increase induced by 50 μM clomiphene was inhibited by 80±5% by 10 μM nifedipine, but was insensitive to 50 μM La^{3+} or 10 μM verapamil. In Ca^{2+}-free medium, pretreatment with 50 μM brefeldin A (to disrupt the Golgi complex Ca^{2+} store), 1 μM thapsigargin (to inhibit the endoplasmic reticulum Ca^{2+} pump), and carbonylcyanide m-chlorophenylhydrazone (CCCP; to uncouple mitochondria) inhibited 51±3% of 50 μM clomiphene-induced Ca^{2+} release; conversely, pretreatment with 50 μM clomiphene abolished the $[Ca^{2+}]_i$ increase induced by thapsigargin, CCCP, and brefeldin A. The Ca^{2+} release-induced by 50 μM clomiphene was unchanged by inhibition of phospholipase C with 2 μM 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione (U73122). Collectively, the results suggest that clomiphene increased $[Ca^{2+}]_i$ in osteoblast-like cells, by

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releasing intracellular Ca^{2+} in a phospholipase C-independent manner and by causing nifedipine-sensitive Ca^{2+} influx.

Key Word: Ca^{2+} signaling, clomiphene, fura-2, MG63 cells, osteoblasts

Introduction

Due to its estrogen-like activity, clomiphene has been shown to increase the secretion of gonadotropins and estrogens, and therefore, has been used as an inducer of ovulation in women with amenorrhea and ovulatory disorders (1-3). Anti-estrogens can bind specifically to the estrogen receptor, to the typical anti-estrogen specific binding site, and to low density lipoproteins in the plasma (4). However, whether clomiphene exerts its action via a single defined pathway or via multiple unrelated pathways is unclear (5, 6). Adverse effects in patients treated with clomiphene are many, including hot flushes, head ache, constipation, allergic skin reactions, and reversible hair loss (7-9).

The effect of clomiphene on signal transduction in cells is unclear. Ca^{2+} regulation plays a pivotal role in signal transduction. Many endogenous chemicals and exogenous drugs can induce an increase in cytosolic free Ca^{2+} level ($[Ca^{2+}]_i$) (10,11). The $[Ca^{2+}]_i$ increase may be due to Ca^{2+} release from various Ca^{2+} stores and Ca^{2+} inflow via channels. A regulated increase in $[Ca^{2+}]_i$ is a key signal for diverse physio-pathological cellular processes in cells; however, unregulated elevations in $[Ca^{2+}]_i$ may cause cell death (10-12).

In this study, fura-2 was used as a Ca^{2+} probe to measure $[Ca^{2+}]_i$ changes in populations of human MG63 osteoblast-like cancer cells. Clomiphene has been shown to exert many effects on bone cells in vivo (13, 14), including prevention of cancellous bone loss from tibia of ovariectomized rats (15), and prevention of estrogen-deficiency osteopenia elicited by LHRH agonists in the rat (16). However, the effect of clomiphene on $[Ca^{2+}]_i$ in bone cells is unclear.

The results show that clomiphene induced increases in $[Ca^{2+}]_i$ in MG63 cells. The concentration-response relationship was established, and the underlying mechanism of the clomiphene response was evaluated.

Materials and Methods

Cell Culture

MG63 cells obtained from American Type Culture Collection were cultured in modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2-containing humidified air.

Solutions

Ca^{2+} medium (pH 7.4) contained (in mM): NaCl 140; KCl 5; $MgCl_2$ 1; $CaCl_2$ 1.8; Hepes 10; glucose 5. Ca^{2+}-free medium had a formula similar to Ca^{2+}-containing medium except that Ca^{2+} was substituted with 1 mM EGTA. The final solution used in the $[Ca^{2+}]_i$ measurements contained less than 0.1% of solvent (dimethyl sulfoxide or ethanol) which did not affect $[Ca^{2+}]_i$ (n=3).

Optical Measurements of $[Ca^{2+}]_i$

Trypsinized cells (10^6/ml) were loaded with 2 μM of the acetoxymethyl ester form of fura-2 (fura-2/AM) for 30 min at 25°C in Ca^{2+} medium. Cells were washed and resuspended in Ca^{2+} medium before use. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF1503PC spectrofluorophotometer by recording excitation signals at 340 and 380 nm and emission signal at 510 nm at 1-s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 20 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously (17, 18).

Chemicals

The reagents for cell culture were from Gibco. Fura-2/AM was from Molecular Probes. U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl) amino)hexyl)-1H-pyrrole-2,5-dione), U73343 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino) hexyl)-2,5-pyrrolidine-dione) were from BIOMOL. All other reagents were from Sigma.

Statistics

The $[Ca^{2+}]_i$ recordings are typical of 4-6 responses. All values were reported as mean±SEM of 4-6 experiments. Because the data from each experiment were the average responses from 0.5 million cells in the cuvet, the variation among experiments was small. Two means were compared

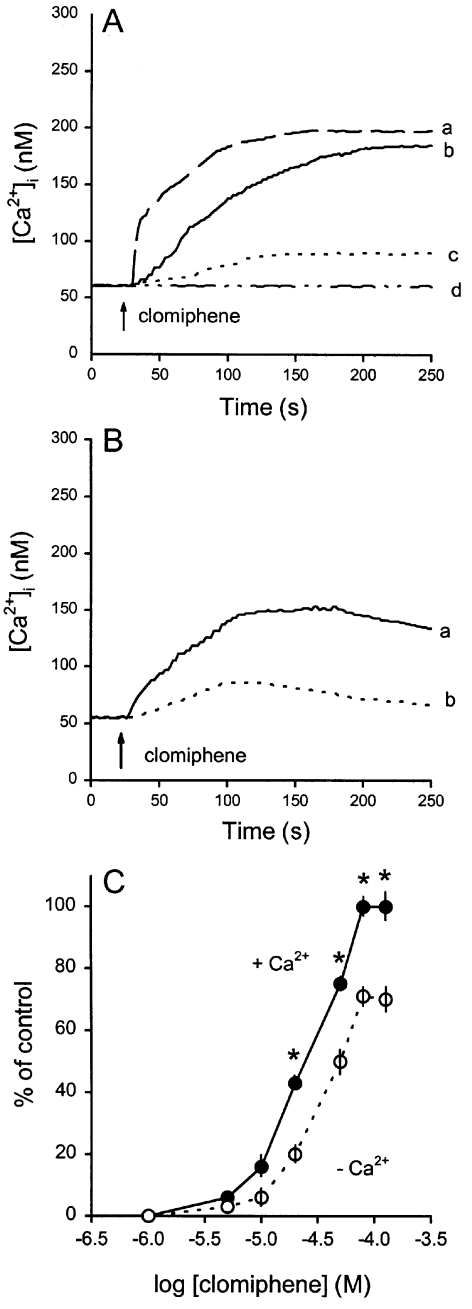

Fig. 1. Effect of clomiphene on $[Ca^{2+}]_i$ in fura-2-loaded MG63 osteoblast-like cells. **A**, Clomiphene-induced $[Ca^{2+}]_i$ increases in Ca^{2+} medium. The drug was added at the time indicated by the arrow. Concentrations of clomiphene were 50 (*trace a*), 25 (*trace b*), 5 (*trace c*) μM, and 1 (*trace d*) μM, respectively. **B**, Effect of removal of extracellular Ca^{2+} on clomiphene-induced $[Ca^{2+}]_i$ increases. The experiments were performed in Ca^{2+}-free medium. Clomiphene was added at 30 s. The concentration of the drug was 50 μM in *trace a* and 25 μM in *trace b*. **C**, Concentration-response plots of clomiphene-induced Ca^{2+} signals in the presence (*filled circles*) and absence (*open circles*) of Ca^{2+}. The y axis is the percentage of control. Control was defined as the net (*baseline subtracted*) maximum $[Ca^{2+}]_i$ induced by 100 μM clomiphene in Ca^{2+}-containing medium. Data were the mean±SEM of 4-6 experiments. *P<0.05 between filled circles and open circles. Traces were typical of 4-6 experiments.

using Student's t-test, and significance was accepted when P<0.05.

Results

Effect of Clomiphene on $[Ca^{2+}]_i$

In Ca^{2+} medium, clomiphene at concentrations between 5-75 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner. Figure 1A (trace a) shows a typical record of the $[Ca^{2+}]_i$ increase induced by 50 μM clomiphene. Over a time period of 5 min the $[Ca^{2+}]_i$ signal consisted of an initial rise which reached a net maximum value of 142±5 nM (baseline subtracted; n=5). The Ca^{2+} signal saturated at 75 μM clomiphene because the responses induced by 75 and 100 μM of the drug were indistinguishable. At a concentration of 1 μM clomiphene hardly had an effect (trace d). Figure 1C (filled circles) shows the dose-response curve of the clomiphene response. The curve suggests an EC50 value of about 50 μM.

Effect of Extracellular Ca^{2+} Removal on the Clomiphene Response

Figure 1B shows that in Ca^{2+}-free medium (no added Ca^{2+} plus 1 mM EGTA to chelate residual Ca^{2+}), 50 μM clomiphene induced an increase in $[Ca^{2+}]_i$ with a net maximum of 142±4 nM (trace a; n=5). The dose-response curve of clomiphene-induced $[Ca^{2+}]_i$ increases in the absence of Ca^{2+} is shown in Figure 1C (open circles). The data suggest that the $[Ca^{2+}]_i$ increases induced by 10-75 μM clomiphene in Ca^{2+} medium was inhibited by extracellular Ca^{2+} removal by 40±10% (n=4-6; P<0.05) in the net maximum value.

Effect of Ca^{2+} Entry Blockers on Clomiphene-Induced $[Ca^{2+}]_i$ Increases

Figure 2 shows that pretreatment with the L-type Ca^{2+} channel blocker nifedipine (10 μM) inhibited 805% of 50 μM clomiphene-inudced net maximum $[Ca^{2+}]_i$ increases in Ca^{2+} medium (n=5; P<0.05). The nonselective Ca^{2+} entry blocker La^{3+} (50 μM) and another L-type Ca^{2+} channel blocker verapamil (10 μM) failed to inhibit clomiphene-induced $[Ca^{2+}]_i$ increases (n=6; P>0.05).

Intracellular Sources of the Clomiphene Response

Figure 3A shows that in Ca2+-free medium, addition of 1 μM thapsigargin, an endoplasmic reticulum Ca^{2+} pump inhibitor (19), induced a significant $[Ca^{2+}]_i$ increase with a net maximum of 51±4 nM (n=4; P<0.05). This suggests that

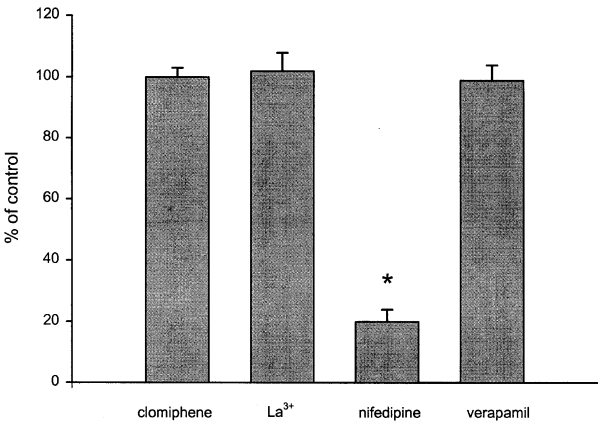

Fig. 2. Effect of Ca^{2+} influx blockers on clomiphene-induced $[Ca^{2+}]_i$ increases. The experiments were performed in Ca^{2+} medium. Cells were stimulated with 50 μM clomiphene in the absence and presence of La^{3+} (50 μM), nifedipine (10 μM) or verapamil (10 μM). The control response was shown as the first column from the left. The clomiphene response in the presence of the blocker was expressed as the percentage of control. Control was defined as the net maximum $[Ca^{2+}]_i$ increases induced by clomiphene in the absence of the blocker. *P<0.05 compared to control. Data were the mean±SEM of 4-6 experiments.

thapsigargin released intracellular Ca^{2+} from the endoplasmic reticulum. After this Ca^{2+} store was depleted by thapsigargin for 6 min, addition of 50 μM clomiphene induced a $[Ca^{2+}]_i$ increase with a net maximum of 58±3 nM which was 59(2% of the control shown in Figure 3D (98±3 nM; n=4; P<0.05). Figure 3B shows that in Ca^{2+}-free mdeium, addition of 2 μM CCCP (a mitochondrial uncoupler) induced a $[Ca^{2+}]_i$ increase with a net maximum of 20±2 nM (n=5). Subsequently added 1 μM thapsigarhin and 50 μM clomiphene induced $[Ca^{2+}]_i$ increases with net maximum values of 42±2 nM and 46±3 nM, respectively (n=4). Similarly, Figure 3C shows that pretreatment with 50 μM brefeldin A to deplete the Ca^{2+} stores in the Golgi complex (20) for 2 min induced a small increase in $[Ca^{2+}]_i$. Subsequently added 2 μM CCCP and 1 μM thapsigargin induced significnat increases in $[Ca^{2+}]_i$ with net maximum values of 20±1 nM and 30±3 nM, respectively (n=4). Notably, after pretreatment with these three drugs, addition of 50 μM clomiphene still induced an increases in $[Ca^{2+}]_i$ with a net maximum of 28±3 nM (n=4). In contrast, Figure 3D shows that after pretreatment with 50 μM clomiphene for 4 min, addition of CCCP, thapsigargin or brefeldin A failed to increase $[Ca^{2+}]_i$ (n=4).

Effect of Inhibition of Phospholipase C on Clomiphene-Induced Ca^{2+} Release

Figure 4A shows a typical $[Ca^{2+}]_i$ increase induced by 10 μM histamine, a phospholipase C-dependent Ca^{2+} mobilizer, in Ca^{2+}-free medium. The $[Ca^{2+}]_i$ signal had a net peak value of 140±4 nM (n=5). Figure 4B shows that after incubation with 2 μM U73122, a phospholipase C inhibitor useful for blocking inositol 1,4,5-trisphosphate formation (21), for 3 min, 10 μM histamine did not cause an increase in $[Ca^{2+}]_i$. U73343 (10 μM), an inactive U73122 analogue that does not inhibit phospholipase C (20), had no effect (n=4; not shown). This suggests that U73122 effectively inhibited the activity of phospholipase C. Subsequently added 50 μM clomiphene (at 340 s) induced a $[Ca^{2+}]_i$ increase with a net maximum value of 101±3 which was similar to the control clomiphene effect (Figure 3D; 1023 nM; n=5).

Discussion

This study has, for the first time, found that clomiphene can induce an increase in $[Ca^{2+}]_i$ in human osteoblasts. Our data suggest that this clinically useful ovolution-inducing drug caused an immedaite and significant increase in $[Ca^{2+}]_i$ in a concentration-dependent manner starting at a concentration of 5 μM, with an EC50 of 50 μM.

The Ca^{2+} signal induced by clomiphene was mainly due to intracellular Ca^{2+} release because removal of extracellular Ca^{2+} only inhibited a small portion of the signal. The intracellular Ca^{2+} stores from which clomiphene releases Ca^{2+} may comprise multiple pools including the endoplasmic reticulum, the mitochondria and the Golgi complex. This is because in Ca^{2+}-free medium, pretreatment with thapsigargin, CCCP and brefeldin A to mobilize Ca^{2+} from these compartments, respectively, inhibited the clomiphene-induced $[Ca^{2+}]_i$ increase in an additive manner, and conversely pretreatment with clomiphene abolished the $[Ca^{2+}]_i$ increase induced by thapsigargin, CCCP or brefeldin A. How clomiphene releases Ca^{2+} from these pools is unclear, but the data suggest that the release seems to be independent of the phospholipase C/inositol 1,4,5-trisphosphate system because clomiphene still released a significant amount of Ca^{2+} when the activity of phospholipase C was suppressed. The clomiphene-induced Ca^{2+} influx was examined and it was found that the influx was sensitive to nifedipine but was insensitive to verapamil. Since nifedipine and verapamil bind to different sites on L-type Ca^{2+} channels, the data suggest that clomiphene may open L-type Ca^{2+} channels by binding to the nifedipine-sensitive, verapamil-insensitive site. The lack of inhibition of the general Ca^{2+} entry blocker La^{3+} could be because that La^{3+} also inhibited Ca^{2+} efflux leading to an increase in $[Ca^{2+}]_i$ and, thus, masked the inhibitory effect on Ca^{2+} influx.

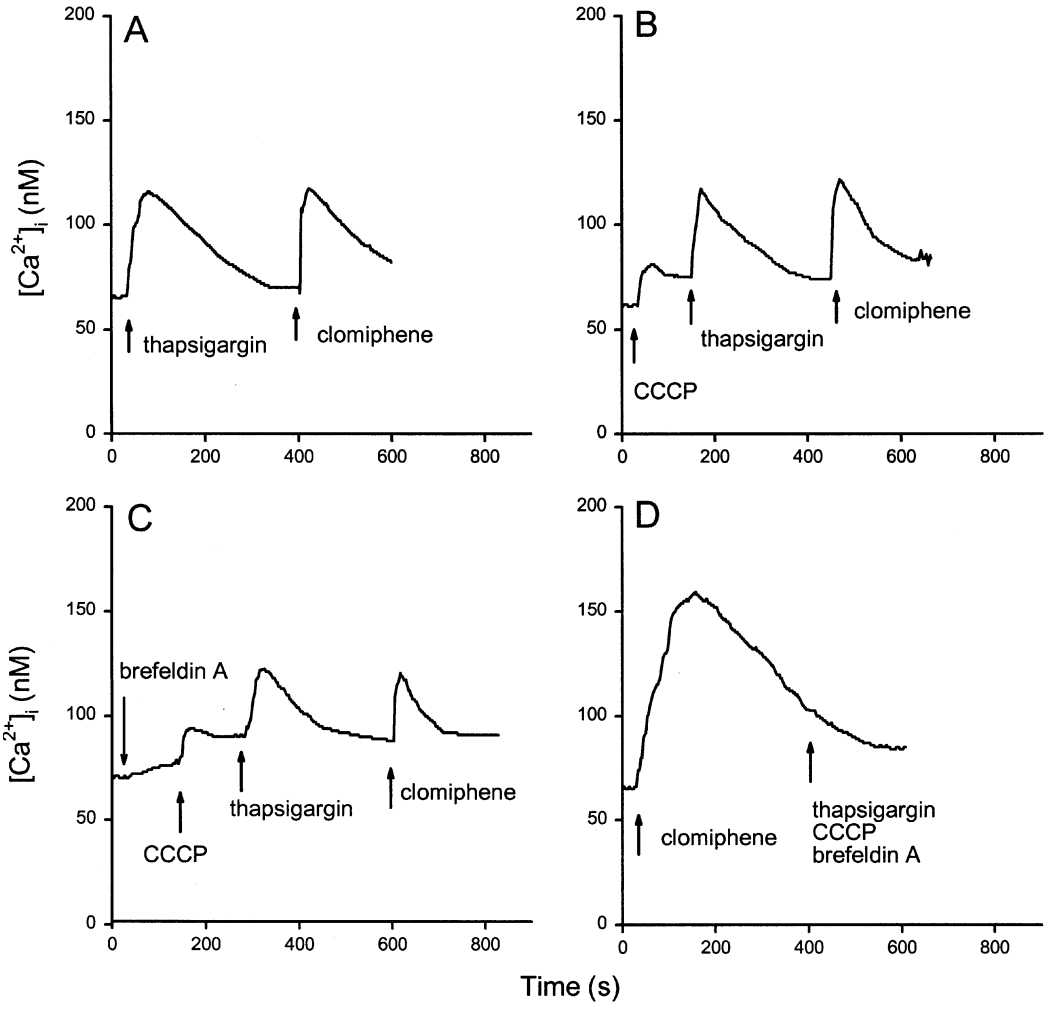

Fig. 3. Ca^{2+} stores of clomiphene-induced Ca^{2+} release. The experiments were performed in Ca^{2+}-free medium. **A**, 1 μM thapsigargin and 50 μM clomiphene were added at the time points shown by arrows. **B**, 2 μM CCCP, 1 μM thapsigargin and 50 μM clomiphene were added as shown. **C**, 50 μM brefeldin A, 2 μM CCCP, 1 μM thapsigargin and 50 μM clomiphene were added as shown. **D**, 50 μM clomiphene was added at 30 s followed by a combined addition of 2 μM CCCP, 1 μM thapsigargin and 50 μM brefeldin A at 400 s. Traces were typical of 4-6 experiments.

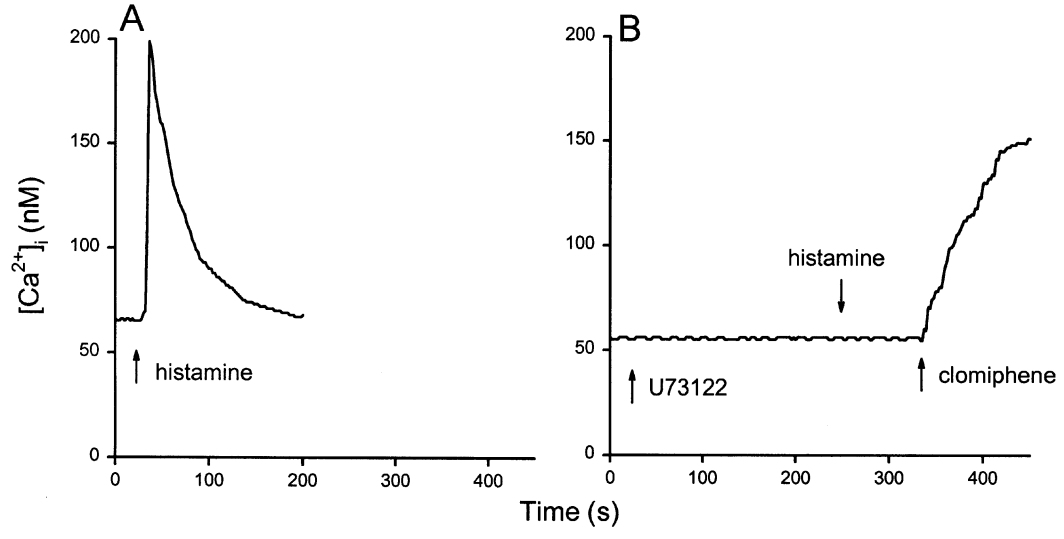

Fig. 4. Effect of inhibition of phospholipase C on clomiphene-induced $[Ca^{2+}]_i$ increases. The experiments were performed in Ca^{2+}-free medium. **A**, 10 μM histamine was added at 20 s. **B**, 2 μM U73122, 10 μM histamine and 50 μM clomiphene were added as shown. Traces were typical of 4-6 experiments.

Due to the rapidity of the clomiphene-induced $[Ca^{2+}]_i$ increase, it is unlikely that this action of clomiphene is mediated by genomic estrogen recpetor activation. Because the concentration of clomiphene in patients taking large doses of the drug may reach μM ranges (22), our data suggest that the possible effect of clomiphene on Ca^{2+} regulation in osteoblasts should be taken into account in evaluating the clinical action and side effect of this drug.

Acknowledgments

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