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Effect of the selective protein kinase C inhibitor, Ro-31-8220, on chemokine-induced Leukocyte recruitment *in vivo*

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Abstract:

The most critical and most important event in acute inflammation is the migration of neutrophils and other inflammatory cells from blood to the site of injury, immune response or infection. Leukocyte recruitment occurs in response to pro-inflammatory mediators such as cytokines and interleukins which are produced at the site of inflammation. Protein kinase C (PKC) is a family of kinases that are involved in the pathophysiology of a variety of inflammatory diseases or disorders such as arthritis, asthma and myocarditis. The effect of Ro-31-8220, the selective PKC inhibitor, on leukocyte transmigration in various inflammatory models is still incompletely understood. The present study explored the effect of the selective and pan inhibitor of PKC, Ro-31-8220, on CXCL1/KC induced leukocyte recruitment especially neutrophils in acute peritonitis model in mice. Ro-31-8220 treatment significantly attenuated the emigration of leukocytes predominately neutrophils in response to CXCL1/KC chemokine. Thereby, Ro-31-8220 treatment ameliorated CXCL1/KC induced acute peritonitis by interfering with emigration of leukocytes. Collectively, our study demonstrates that pharmacological inhibition of PKC in general, may provide the basic key of therapeutic strategy for many inflammatory diseases or immune linked disorders in which PKC was implicated.

Keywords: inflammation, leukocytes, neutrophils, PKC, Ro-31-8220, peritonitis,CXCL1/KC chemokine.

1. Introduction

Inflammation is a natural body defense mechanism against various types of pathogens and injuries.Although inflammation is crucial for survival, excessive inflammation can also be harmful, leading to extensive tissue damage that resulting in systemic inflammatory responses or multiple organ failure. Therefore, limitation of exaggerated inflammatory response toward pathogen is also essential to

save body tissue and organs and this can be achieved by either reducing the number of emigrated leukocytes to site of injury or infection or by reducing production of pro-inflammatory mediators such as cytokines and interleukins(1,2,3,4) .Among the most critical steps in acute inflammation is the migration of neutrophils andother inflammatory cells from the circulation to the infection sitethrough vascular endothelium of postcapillaryvenules(5,6,7).Neurtophils (Polymorphonuclear leukocytes) are considered the first defense line against different pathogens such as bacteria, protozoa and fungi. They are well recognized as the hallmark feature of acute inflammation because they are the first and the dominant leukocytes that migrate to the site of acute inflammation. They possess awide range of antimicrobialactivities including killing and phagocytosis of foreign pathogens, generation of oxygen free radicals and production of microbicidalenzymes and inflammatory interleukins. Therefore, they exert a crucial role in inflammation(8,9,10). Neutrophil recruitment to the site of infection or injury occurs in response to pro-inflammatory mediators which areinterleukins such as IL8 and cytokines such as CXCL1, CXCL2 and CXCL5 chemokines.These neutrophil chemoattractantsare produced at the site of inflammation by various inflammatory cells (39).

Protein kinase C (PKC) family is comprised of at least 11 phospholipid-dependent serine/threonine kinases which are similar in their catalytic kinase domains and cysteine rich regions.Each PKC isozyme consists of a single polypeptide chain that conserving two crucial functional domains: aC-terminal protein kinase domain and N-terminal regulatory domain(11-12).

This family of protein kinases is subdivided into conventional, novel and atypical isoforms. The conventional group includes four isozymes which are PKCα, PKCβ I, PKCβ II and PKCɣwhile the novel group includes four isozymes which are PKCδ, PKCε, PKCη and PKCθ. The last group, atypical isozymes, comprised of PKCζ and PKCι (13-15). These PKC isozymes are activated by G-protein coupled receptors (GPCRs), tyrosine kinase receptors and growth factor receptors.The agoniststhat hydrolyze cell membrane phosphoinositide lead to generation of inositol triphosphate (IP3) and diacylglycerol (DAG). Production of IP3 promote intracellular calcium mobilization.Both of calcium (Ca^{+2}) and DAG intermediate activation of PKC isoforms. The activated PKC regulate various biological functions in different cells through phosphorylation of its target proteins(16-17). It has been shown that PKC is involved in modulation of cellulargrowth,

differentiation,proliferation and activation. In addition to that, it is also involved in regulation of inflammatory responsesbecause it is contributed in induction process of inflammatory mediators release such as chemokines, and other lipid mediators (18-21). PKC isoforms are ubiquitously expressed in different cell types(17). Among these cells in which PKC is expressed are haematopoietic cells: neutrophils, lymphocytes,
monocytes,macrophages, mast cells and platelets(11,25-26). PKC has been involved in pathogenesis of many diseases and organ dysfunctions such as diabetes, hypertension, oxidative stress, cardiomyopathy, fibrosis, thrombosis, cancer, autoimmune diseases and other inflammatory disorders(22-25).

The bisindolylmaleimideRo-31-8220 is a widely used selective inhibitor of the PKC and binds to the active site of PKC enzyme in an ATP-competitive manner(36-38).Since PKC is expressed in leukocytes and implicated in various inflammatory related diseases or disorder, the purpose of this study was to elucidate the effect of PKC pan inhibitor, Ro-31-8220, on the number of emigrated leukocytes mainly neutrophils in chemokine induced acute peritonitis in mice. This elucidation might provide the basic key for therapeutic intervention in miscellaneous inflammatory diseases in which PKC was implicated.

2. Material and methods

2.1 Chemicals

the CXC keratinocyte-derived chemokine (KC/CXCL1) were obtained from Peprotech, Rocky Hill, USA and Ro-31-8220 were purchased from Cayman Chemical, Michigan, USA.All other used chemicals and reagents were of analytical grade.

2.2 Animals

C57BL/6 male mice aged between 8-16 week-old were used in this study. All procedures were approved by the University Committee on Animal Care and Supply (UCACS) at the University of Saskatchewan and met the standards of the Canadian Council on Animal Care.

2.3 KC/CXCL1-induced peritonitis

To induce acute mouse peritonitis, the CXC keratinocyte-derived
chemokine (KC/CXCL1, 0.51 µg) in 300 µl of sterile saline was injected intraperitoneally (i.p.) into each C57BL/6 WT mouse. The mice were divided into two groups which are the control group and the Ro-31-8220 treated group. The control group mice were received ani.p injection of 2%dimethyl sulfoxide (DMSO,Santa Cruz Biotechnology, Texas, USA) 1 hour before KC injection while mice of the second group were

given ani.p. injection of Ro-31-8220 1hour before KC administration in a doseof 6 mg/kg based on previously reported optimal concentrations (27).The mice of both groups were given free access to water and food for 4 hours. Mice were euthanized after 4 hours of KC injection by cervical dislocation. Thereafter, the peritoneal lavage was performed using 10 ml of cold phosphate-buffered saline (PBS) containing0.25% BSA and 0.02% EDTA; (pH 7.35). Then, the collected peritoneal lavage fluid from each mouse was centrifuged separately for 6 min at 1300 rpm , 4 °C and the supernatant was discarded and cell pellet wasresuspended in 0.5 – 1.5ml of cold PBS.The total number of leukocytes was determined by optical microscopy in Hemocytometer, using Turk's solution (0.01% crystal violet in 3% acetic acid) whilethe differential cell countwas performed by counting at least 300 cells on cytospinslides stained with H&Estain and then differentiating them by standard morphological criteria. The total numbers of neutrophils lymphocytes and monocytesin the peritoneal cavity were then calculated for both animal groups (control group and Ro-31-8220).

2.4 Data analysis

Data in this study were analyzed usingExcel software program andexpressed as mean ± SEM. *p* Values were calculated usingtwo-tailed Student's *t* test. *p* Values < 0.05 were considered statistically significant.*n* indicates the number of mice used in each group.

3. Results

1. Effect of CXCL1/KC chemokine on neutrophil recruitment

Our experimental model demonstrated that CXC chemokine KC/CXCL1 is a very potent chemoattractant for neutrophils becausemore than 80% of the emigrated leukocytes were found to be neutrophils in response to this chemokine (Table-1 and Figure 1). This result confirmed theresults of previous studies (28-30).

Table-1: The percentage of different leukocyte subtypes emigrated to the peritoneum after 4 hours of an i.p. injection of CXCL1/KC chemokine (0.51 µg/mouse) in the control group.

No of mice	Neutrophil %	Lymphocyte %	Monocyte %	Other granulocytes %
Mouse 1	80.5	16	2.8	0.7
Mouse 2	85.21	13	1.25	0.54
Mouse 3	79.03	19.01	1.33	0.63
Mouse 4	87.13	11.22	1.15	0.5
Average	82.9675 ± 1.91	14.8075 ± 1.71	1.6325 ± 0.39	0.5925 ± 0.04

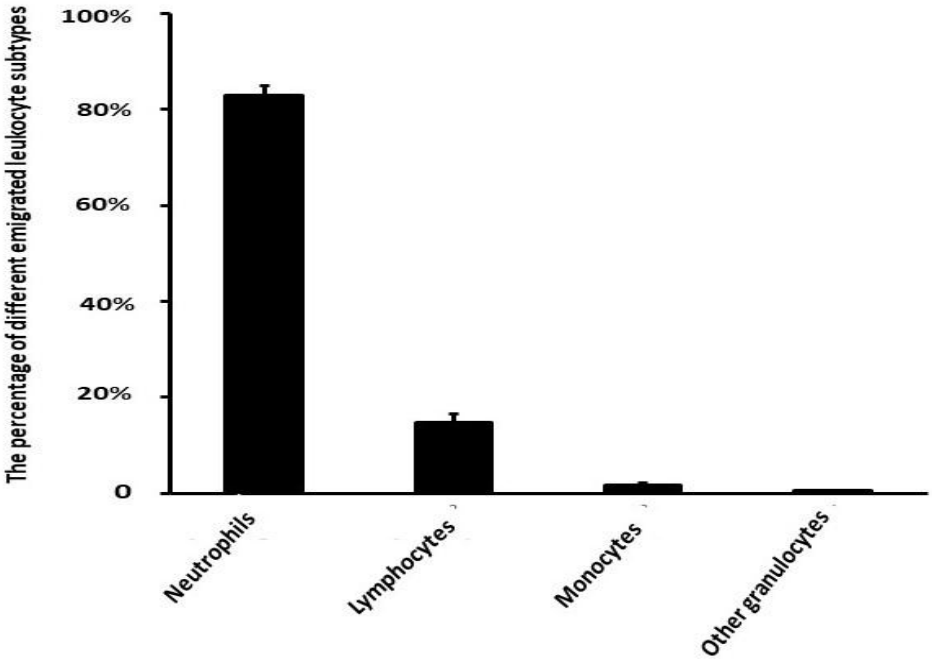

Figure1. The percentage of different leukocyte subtypes emigrated to peritoneum after 4 hour of intraperitoneal injection of CXCL1/KC chemokine (0.51 µg/mouse) in the control group**.** Data are means ± SEM (*n* = 4).

2. Effect of Ro-31-8220 treatment on emigrated leukocytes

2.1 The suppressant effect of Ro-31-8220 treatment on the total number of emigrated leukocytes.

We performed a series of experiments to explore the effect of Ro-31-8220 treatment on KC-triggered infiltration and emigration of leukocytes with their differential subtypes to the peritoneal cavity (KC-induced acute peritonitis). As shown in Table-2 and Figure2A, Ro-31-8220 treatment significantly reduced the number of emigrated leukocytes in the peritoneal lavage fluid after 4 hours of KC injection in comparison to control group (p value < 0.01).

2.2 The suppressant effect of Ro-31-8220 treatment on emigration of neutrophils and lymphocytes

Moreover, after we have analyzed the differential counts of emigrated leukocytes to the peritoneum, we found that Ro-31-8220 treatment causes significant reduction in the number of emigrated neutrophils and

lymphocytes to the peritoneum in comparison to control group, as shown in Table-2 and Figure2B and 2C respectively. (p value < 0.001 for neutrophils and <0.05 for lymphocytes).

Table-2: The number of emigrated leukocytes, neutrophils and lymphocytes counted in the peritoneal lavage fluid in both the control group and Ro-31-8220 treated group after 4 hours of an i.p. injection of CXCL1/KC chemokine (0.51 µg/mouse) .

No. of mice	Total leukocytes count ($\times 10^6$ cells)		Neutrophils count ($\times 10^6$ cells)		Lymphocytes count ($\times 10^6$ cells)	
	Control	Ro-31-8220	Control	Ro-31-8220	Control	Ro-31-8220
Mouse 1	10.2	4.1	8.21	3.49	1.63	0.496
Mouse 2	7	3.15	5.96	2.48	0.91	0.550
Mouse 3	11.5	3.9	9.08	3.40	2.18	0.430
Mouse 4	8.75	3.53	7.62	2.89	0.98	0.544
Average	9.36 ± 0.97	3.67 ± 0.21	7.72 ± 0.66	3.06 ± 0.23	1.42 ± 0.30	0.50 ± 0.027

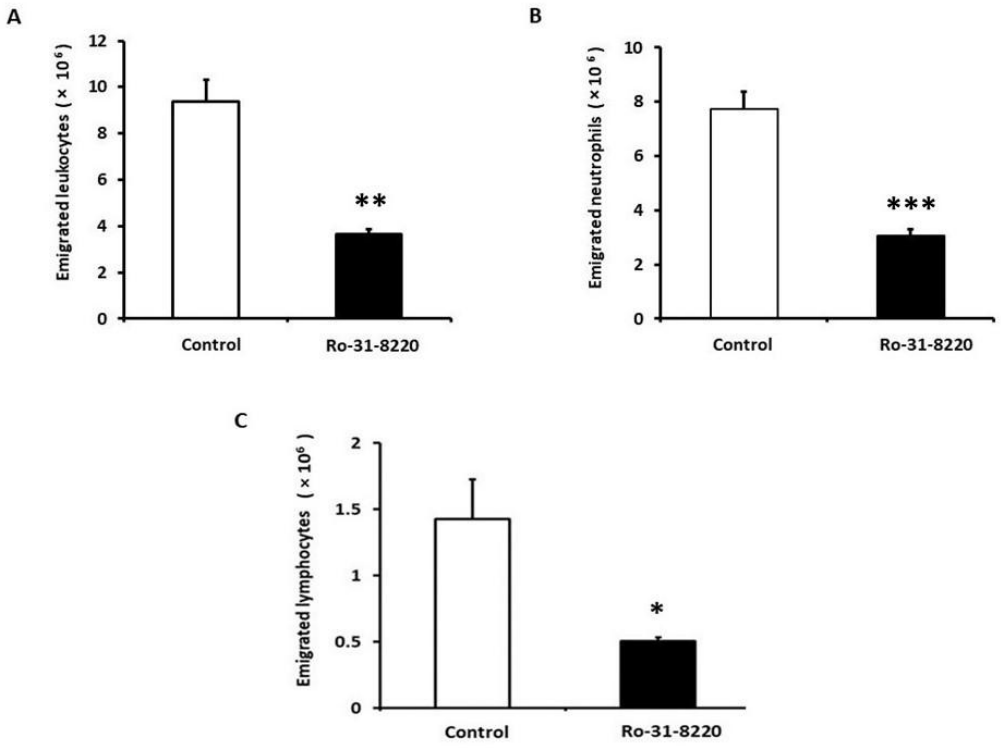

Figure 2. Effect of Ro-31-8220 on CXCL1-induced leukocyte emigration in peritoneum. **(A)** the number of emigrated leukocytes, **(B)** the number of emigrated neutrophils and **(C)** the number of emigrated lymphocytes ($\times 10^6$ cells) counted in the peritoneal lavage fluid in the absence (Control) and in the presence of PKC selective inhibitor Ro-31-8220 (6 mg/kg, i.p. 1 hour prior to CXCL1 injection) collected after 4 hours of an i.p. injection of CXCL1 (0.51 µg/mouse)**.** Data are means ± SEM (n = 4).*, ** and *** indicatesignificant difference (p< 0.05, p < 0.01and p < 0.001, respectively) fromthe control.

4. Discussion

The present study disclosesthe effect of pharmacological inhibition of PKC on chemokine CXCL1-inducedleukocyte recruitment especially neutrophils. We showedthat the predominant subtype of leukocytes recruited to site of acute inflammationin response to CXC chemokine KC was neutrophils. Furthermore,we explored thatPharmacological inhibition of PKC by using the selective PKC inhibitorRo-31-8220 attenuatedKC/CXCL1-induced transmigration of leukocytes predominately neutrophils from blood to inflamed peritoneal tissue.

Therefore, our finding provides a crucial support for previous studies that highlighted the pro-inflammatory role of PKC in various models. It has been reported that PKC activation was participated in pathogenesis of arthritis, asthma, lung inflammation and acute respiratory distress syndrome (32-34). Robert Gray and coworkers reported the significant role of PKC in formation of neutrophil extracellular trap (NET); a mechanism by which neutrophils catch and kill the microorganisms. NET formation was blocked by pharmacological inhibition of PKC in neutrophils usingRo-31-8220 and other PKC inhibitors (13). In addition to that, PKC activation was found to be a strong activator of neutrophil NADPH oxidase enzyme which is responsible for production of oxygen free radicals that kill the phagocytosed bacteria or microbes in a process termed an oxidative burst or respiratory burst (35).Heiskanen et al.demonstrated that Palmitic acid anilide-induced respiratory burst in human neutrophils is inhibited by a protein kinase C inhibitor, Ro 31-8220 (41).

Moreover, some studies documented that PKC activation enhances neutrophil adhesion to endothelium of blood vessel which is a preceding step to the transmigration step to the inflamed interstitium, thereby demonstrating the pro-inflammatory role of PKC(31). Although that, the effect of Ro-31-8220, the selective PKC inhibitor, on leukocyte transmigration especially neutrophils in various inflammatory models is still incompletely understood because some studies documented that*in vitro*formyl-methionyl-leucyl-phenylalanine (fMLP)-induced neutrophil migration was highly increased in presence of Ro-31-8220 but Galectin-1 induced neutrophil migration was highly impaired by Ro-31-8220 (40).Furthermore, Berger C.and coworkers showed that chemokine induced neutrophil adhesion and transmigration was significantly reduced by Ro 31-8220 (42). Thereby, it is clear that the effect of thePKC selective inhibitor,

Ro 31-8220, on neutrophil transmigration is still controversial and elusive. Therefore, we conducted this *invivo* experimental research to demonstrate the effect of Ro-31-8220 on neutrophil transmigration in acute peritonitis model.

Taken together, our results confirmed the pro-inflammatory effect of PKC in a different inflammatory model which is CXC chemokine KC induced acute peritonitis model. It is due to that pharmacological inhibition of PKC by Ro-31-8220 produced a suppressant effect on inflammatory process by decreasing the number of emigrated leukocytes especially neutrophils to the site of acute inflammation.

5. Conclusion

pharmacological suppression of PKC byRo-31-8220 significantly impairs leukocyte recruitment especially neutrophil *in vivo.* Therefore, Pharmacological inhibition of PKC in general, mayprovide the basic key of therapeutic strategy for manyinflammatory diseases or immune linked disorders in which PKC was implicated.

Conflicts of Interest

Authors declare no conflict of interest

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