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Precision-cut tissue slices: a novel ex vivo model for fibrosis research

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Chapter 5

Organ- and species-specific biological activity of
rosmarinic acid

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ABSTRACT

Background: Rosmarinic acid (RA), a compound found in several plant species, has beneficial properties, including anti-inflammatory and antibacterial effects. We investigated the toxicity, anti-inflammatory, and antifibrotic effects of RA using precision-cut liver slices (PCLS) and precision-cut intestinal slices (PCIS) prepared from human, mouse, and rat tissue.

Methods: PCLS and PCIS were cultured up to 48 h in the absence or presence of RA. Gene expression of the inflammatory markers: *IL-6*, *IL-8*, *CXCL1/KC*, and *IL-1 β* , as well as the fibrosis markers: *pro-collagen 1a1*, *heat shock protein 47*, α -smooth muscle actin, *fibronectin* and *plasminogen activator inhibitor-1* were evaluated by qPCR.

Results: RA was only toxic in murine PCIS. RA failed to mitigate the inflammatory response in most models, while it clearly reduced *IL-6* and *CXCL1/KC* gene expression in murine PCIS at non-toxic concentrations. With regards to fibrosis, RA decreased the gene levels of *Fn2* and *Pai-1* in murine PCLS, and *Fn2* in murine PCIS. Yet, no effect was observed on the gene expression of fibrosis markers in human and rat PCIS.

Conclusions: In conclusion, we observed clear organ- and species-specific effects of RA. RA had little influence on inflammation. However, our study further establishes RA as a potential candidate for the treatment of liver fibrosis.

INTRODUCTION

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyl-lactic acid (Figure 1). It is commonly found in plants of the Boraginaceae family (forget-me-not) and the subfamily Nepetoideae of the mint family Lamiaceae. It is also found in some fern and hornwort species (Petersen and Simmonds, 2003). RA has a gamut of beneficial biological activities, such as anti-inflammatory (Rocha et al., 2015), antioxidant, antiapoptotic, and antifibrotic effects (Domitrović et al., 2013; Li et al., 2010).

Rocha et al. demonstrated that RA might be useful in the pharmacological modulation of injuries associated with inflammation (Rocha et al., 2015). The anti-inflammatory properties of RA are thought to be based on the inhibition of lipoxygenase and cyclooxygenases, interference with the complement cascade (Krol et al., 1996; Mirzoeva and Calder, 1996; Petersen and Simmonds, 2003) and down-regulation of inflammatory cytokines (Sanbongi, 2003) which was characterized by neutrophil sequestration and interstitial edema. DEP enhanced the lung expression of keratinocyte chemoattractant (KC). Because chronic inflammation is an important trigger for fibrogenesis, RA might mitigate fibrosis by dampening the inflammatory response during chronic diseases. Liver fibrosis, especially the end stage cirrhosis, is a major cause of mortality worldwide (Poynard et al., 2010) identifying independent risk factors and to propose screening strategies in the general population. METHODS: We prospectively studied 7,463 consecutive subjects aged 40 years or older. Subjects with presumed advanced fibrosis (FibroTest greater than 0.48). Similarly, intestinal fibrosis is found in most patients with inflammatory bowel disease (IBD), which affects at least 2.2 million Europeans (Loftus, 2004; Poynard et al., 2010). Currently, the only available treatment for liver and intestinal fibrosis is surgery, therefore there is an urgent need for alternative and effective treatment modalities.

Previously, Westra et al. showed that RA decreased the expression of the fibrosis markers *collagen 1a1* (*Col1a1*), *heat shock protein 47* (*Hsp47*), and α -smooth muscle actin (α *Sma*) in both human and rat precision-cut liver slices (Westra et al., 2014a). In addition, RA also showed therapeutic activity against acute liver toxicity *in vivo* (Li et al., 2010). RA ameliorated hepatic oxidative/nitrosative stress, suppressed inflammation, and inhibited activation of hepatic stellate cells (HSCs) and apoptosis in CCl₄-injured livers. The hepatoprotective activity of RA was accompa-

nied by induction of the Nrf2/HO-1 pathway (Domitrović et al., 2013). Moreover, it has also been shown that RA inhibits COX-2 activation in colon cancer HT-29 cells (Hossan et al., 2014; Scheckel et al., 2008). These results suggest that RA may be a promising anti-inflammatory and antifibrotic compound in both liver and intestinal fibrosis. Yet, the discovery of effective antifibrotics is hampered by the absence of good translational models, variability in the observed efficacy of drug candidates in rodent models due to species- and strain-dependent responses and the inability to replicate the multicellular pathophysiology of fibrosis *in vitro* (Mutsaers et al., 2015; Torok et al., 2015; Westra et al., 2013). To tackle these issues, the objective of this study was to investigate organ- and species-specific biological activity of RA with a focus on the putative anti-inflammatory and antifibrotic effects using precision-cut liver slices (PCLS) and precision-cut intestinal slices (PCIS) prepared from human, murine, and rat tissue.

This *ex vivo/in vitro* model is ideal to study multicellular processes, *e.g.* fibrosis, because the slices contain all the different cells in their original environment allowing for cell-cell and cell-matrix interactions (Baumgart and Carding, 2007).

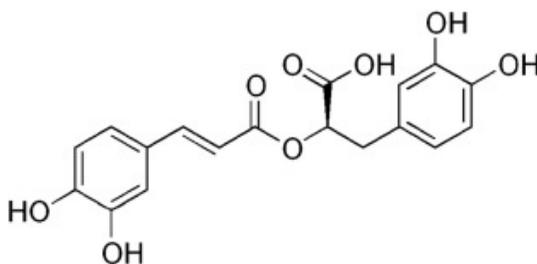


Figure 1. Rosmarinic acid structure

MATERIALS AND METHODS

Chemical

All chemicals were obtained from Sigma (Zwijndrecht, the Netherlands) unless stated otherwise. Stock solutions of RA were prepared in milli-Q and stored at -20°C . During experiments, stocks were diluted in culture medium with a final solvent concentration of $\leq 1\%$.

Animals

Tissue was obtained from male Wistar rats (Harlan Laboratories B.V., Horst, The Netherlands) and C57BL/6 mice (De Centrale Dienst Proefdieren, University Medical Center Groningen, Groningen, The Netherlands). Animals were housed under controlled conditions with a twelve-hour light/dark cycle and free access to water and food (Harlan chow no.2018, Horst, The Netherlands). Organs were harvested via a terminal procedure performed under isoflurane/O₂ anesthesia (Nicholas Piramal, London, UK). All experiments were approved by the Animal Ethical Committee of the University of Groningen.

Preparation of murine precision-cut liver slices

Murine liver slices (PCLS) were prepared according to the protocol by de Graaf et al. (de Graaf et al., 2010). In short, liver cores were obtained using a 5-mm biopsy-punch. Subsequently, slices were made using a Krumdieck tissue slicer (Alabama Research and Development, USA), filled with ice-cold Krebs-Henseleit buffer (KHB) supplemented with 25 mM D-glucose (Merck, Darmstadt, Germany), 25 mM NaHCO₃ (Merck), 10 mM HEPES (MP Biomedicals, Aurora, OH), saturated with carbogen (95% O₂/5% CO₂) and adjusted to pH 7.4. PCLS with a wet weight of approximately 3 mg, have an estimated thickness of 300-400 μm . To prevent rapid loss of viability after slicing, PCLS were directly transferred to ice-cold University of Wisconsin organ preservation solution (UW-solution).

Preparation of intestinal slices

Healthy human jejunum tissue was obtained from pylorus preserving pancreaticoduodenectomies. Use of human tissue was approved by the Medical Ethical Committee of the University Medical Centre Groningen (UMCG), according to Dutch legislation and the Code of Conduct for dealing responsibly with human tissue in the context of health research (www.federa.org), refraining the need of written consent for 'further use' of coded-anonymous human tissue. The procedures were carried out in accordance with the experimental protocols approved by the Medical Ethical Committee of the UMCG.

Rat jejunum (about 25 cm distal from the stomach and 15 cm in length) or mouse jejunum (about 15 cm distal from the stomach and 10 cm in length) were excised and preserved in ice-cold KHB until use.

Slicing of precision-cut intestinal slices

Preparation of intestinal slices (PCIS) was carried out according to the protocol of de Graaf et al (de Graaf et al., 2010). In short, tissue was cleansed by flushing KHB through the lumen and subsequently divided into 2 cm segments. Afterwards, intestinal cores were prepared using 3% (w/v) agarose (Sigma-Aldrich, Steinheim, Germany) in 0.9% NaCl at 37°C and embedded in an agarose core-embedding unit. Next, PCIS were prepared using a Krumdieck tissue slicer. Similar to PCLS, PCIS had a wet weight of approximately 3 mg, and an estimated thickness of 300-400 µm. Following slicing, PCIS were directly transferred to KHB to prevent loss of viability.

Incubation of slices

After slicing, PCLS and PCIS were cultured in 12-well plates or 24-well plates (murine PCIS) in Williams' Medium E + GlutaMAX (Gibco, New York, USA) supplemented with 14 mM Glucose (Merck, Darmstadt, Germany) and 50 µg/ml gentamycin (Gibco). PCIS medium also contained 2.5 µg/ml fungizone (amphotericin B; Invitrogen, Paisley, UK). Slices were incubated for 24 h (rat PCIS) or 48 h at 37°C in an 80% O₂/5% CO₂ atmosphere. The culture plates were horizontally shaken at 90 rpm (amplitude 2 cm). For experiments, PCLS and PCIS were incubated with RA (100 µM - 500 µM) for 24 - 48h.

Viability

Viability of the slices was assessed by measuring the adenosine triphosphate (ATP) content using the ATP bioluminescence kit (Roche diagnostics, Mannheim, Germany), as previously described (de Graaf et al., 2010). Determined ATP values (pmol) were normalized to the total amount of protein (μg) estimated by the Lowry method (BIO-rad RC DC Protein Assay, Bio Rad, Veenendaal, The Netherlands). Values displayed are relative values compared to the related controls.

Gene expression

After incubation, PCLS and PCIS were snap-frozen in liquid nitrogen, and stored at -80°C until use. Total RNA of three to six pooled snap-frozen slices was isolated using the Qiagen RNAeasy mini kit (Qiagen, Venlo, The Netherlands), and the amount of isolated RNA was measured with the BioTek Synergy HT (BioTek Instruments, Vermont, USA). Afterwards, RNA ($1\ \mu\text{g}$) was reverse transcribed using the reverse transcriptase kit (Promega, Leiden, The Netherlands). The RT-PCR reaction was performed in the Eppendorf mastercycler using the following gradient: 25°C for 10 min, 45°C for 60 min and 95°C for 5 min.

Subsequently, gene expression was studied via qPCR using the SYBR green method or TaqMan gene expression assays (Applied Biosystems, Bleiswijk, The Netherlands). Samples were analyzed using a 7900 HT Fast Real-Time RT-PCR (Applied Biosystems) with 45 cycles of 10 min 95°C , 15 s at 95°C , and 25 s at 60°C following by a dissociation stage (SYBR green) or with 40 cycles of 10 min at 95°C , 15 s at 95°C and 1 min at 60°C (TaqMan). *GAPDH* was used as reference gene, and relative expression was calculated as fold change ($2^{-\Delta\Delta\text{Ct}}$). Used primers and probes are listed in Table 1.

Statistics

Statistics were performed using GraphPad Prism 6.0 via one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test or an unpaired, two-tailed Student's t-test as appropriate. A minimum of four different intestines/liver was used for each experiment, using 3–6 slices from each intestine/liver per

condition. The results are expressed as mean \pm standard error of the mean (SEM). Statistical differences in ATP levels were determined using the values relative to the control values. Regarding the qPCR results, statistics were performed using the $\Delta\Delta C_t$ values, while the data is presented as fold change ($2^{-\Delta\Delta C_t}$). Differences between groups were considered to be statistically significant when $p < 0.05$.

Table 1. Fibrotic and inflammatory primers and probes gene expression

Species	Primer	Forward sequence	Reverse sequence	Probe sequence
Human	<i>GAPDH</i>	ACCAGGGTGC TTTAACTCT	GGTGCCATGGAATTGCC	TGCCATCAATGACCCCTTCA
	<i>COL1A1</i>	CAATCACCTGCGTACAGAACGCC	CGGCAGGGCTCGGGTTTC	CAGGTACCATGACCCGAGACGTG
	<i>HSP47</i>	GCCCCCGTGGTGCCCA	GCCAGGGCCGCCTCCAGGAG	CTCCCTCTGCTTCTCAGCG
	<i>αSMA</i>	AGGGGGTGATGGTGGGAA	ATGATGCCATGTTCTATCGG	GGGTGACGAAGCACAGAGCA
	<i>FN2</i>	AGGCTTGAACCAACCTACGGATGA	GCCTAAGCACTGGCACAAACAGTTT	ATGCCGTTGGAGATGAGTGGGAA
	<i>PAI-1</i>	CACGAGTCTTTCAGACCAAG	AGGCAAATGTCTTCTCTTCC	-
	<i>IL-6</i>		Hs00985639_m1	
	<i>IL-8</i>		Hs00174103_m1	
	<i>IL-1β</i>		Hs01555410_m1	
Mouse	<i>Gapdh</i>	ACAGTCCATGCCATCACTGC	GATCCACGACGGACACATTG	-
	<i>Colla1</i>	TGACTGGAAGAGCGGAGAGT	ATCCATCGGTCAATGCTCTCT	-
	<i>Hsp47</i>	AGGTACCAAGGATGTGGAG	CAGCTTCTCTTCTCGTCGT	-
	<i>αSma</i>	ACTACTGCCGAGCGTGAGAT	CCAATGAAAGATGGCTGGAA	-
	<i>Fn2</i>	CGGAGAGAGTGCCTACTA	CGATATTGGTGAATCGCAGA	-
	<i>Pai-1</i>	GCCAGATTATCATCAATGACTGGG	GGAGAGGTGCACATCTTCTCAAAG	-
	<i>IL-6</i>		Mm00446190_m1	
	<i>CXCL1/KC</i>		Mm04207460_m1	
	<i>IL-1β</i>		Mm00434228_m1	
Rat	<i>Gapdh</i>	GAACATCATCCCTGCATCCA	CCAGTGAGCTTCCCCTTCA	CTTGCCACAGCCTTGGCAGC
	<i>Colla1</i>	CCCACCGGCCCTACTG	GACCAGCTTACCCTTAGCA	CCTCCTGGCTTCCCTG
	<i>Hsp47</i>	AGACGAGTTGTAGATCCAAGAGT	ACCCATGTGTCTCAGGAACCT	CTTCCCCTTCCAGCC
	<i>αSma</i>	AGCTCTGGTGTGTGACAATGG	GGAGCATCATCACCAGCAAAG	CCGCCTTACAGAGCC
	<i>Fn2</i>	TCTTCTGATGTCACCCCAACTCA	TGATAGAATCTCTTGGGGCGGCA	-
	<i>Pai-1</i>	AACCCAGGCCGACTTCA	CATGCGGGCTGAGACTAGAAT	-
	<i>IL-6</i>	CCGGAGAGGAGACTTCACAG	ACAGTGCATCATCGTGTTC	-
	<i>IL-8</i>	GGCAGGGATCACTTCAAGA	GCCATCGGTGCAATCTATCT	-
	<i>IL-1β</i>	AGGCAGTGTCACTATTGTG	GGAGAGCTTTCAGCTCACAT	-

RESULTS

Antifibrotic effect of RA in murine PCLS

Our lab previously demonstrated that RA mitigated fibrogenesis in human and rat PCLS. To elucidate potential species differences, we investigated whether the same effect could be observed in murine PCLS (n = 4). First we characterized the viability, by ATP content, and both the inflammatory and fibrotic response in the slices during culture. As shown in Figure 2A (n = 4), ATP content of murine PCLS significantly increased after 24 h of culture, as compared to the 0 h control. Furthermore, ATP levels remained elevated, indicating that the slices were viable for 48 h. In addition, we observed an increase in inflammatory markers. Gene expression of *IL-6* was significantly up-regulated 77 fold after 24 h and *IL-6* levels were further elevated at 48 h (165 fold; Figure 3A, n = 4). Concurrently, qPCR revealed a marked increase in multiple fibrosis markers after 48 h. Gene expression of *Colla1*, *Hsp47*, *Fn2*, and *Pai-1* were significantly elevated 15, 7, 43, and 216 fold, respectively. These results indicate the presence of both an inflammatory and fibrotic response in murine PCLS during culture. Figure 3B (n = 4) demonstrates that RA (100-300 μ M) does not exert toxicity in PCLS, yet we observed a concentration-dependent induction of IL-1 β after 24 h (Figure 3C, n = 4). Conversely, RA effectively mitigated fibrogenesis in PCLS as shown by a clear reduction in the expression of both *Fn2* and *Pai-1* at all the tested concentrations (Figure 3D, n = 4). Thus, despite the observed induction of *IL-1 β* , RA shows great potential as antifibrotic compound in murine PCLS, in concordance with our previous findings in human and rat PCLS (Westra et al., 2014b; Westra et al., 2016).

Antifibrotic effect of RA in human, murine, and rat PCIS

Next, we investigated whether RA had a similar positive effects in intestinal slices prepared from tissue obtained from man, mouse, and rat.

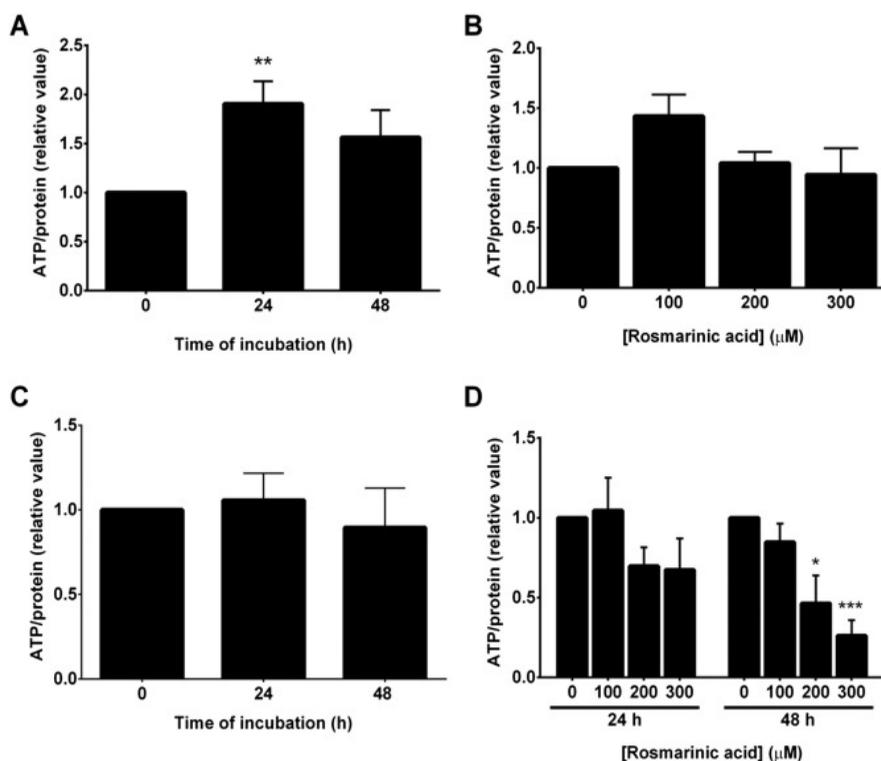


Figure 2. The effect of incubation and rosmarinic acid on murine PCLS ($n = 4$) and PCIS 48 h ($n = 4$): (A) PCLS viability (relative value; incubation); (B) PCLS viability (relative value; rosmarinic acid); (C) PCIS viability (relative value; incubation); (D) PCIS viability (relative value; rosmarinic acid). Data are expressed as mean \pm SEM. Student's t-test; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

Human PCIS

Figure 4A demonstrates that the viability of human PCIS ($n = 9$) remained constant during culture. In addition, we observed a significant up-regulation of *IL-6*, *IL-8*, and *IL-1 β* gene expression (Figure 5A, $n = 5$) as well as elevated *PAI-1* levels during culture (Figure 5B, $n = 5$). The latter is in line with the observed onset of fibrosis in human PCIS reported previously (Pham et al., 2015). These results indicate the presence of both an inflammatory and fibrotic response in human PCIS during culture. As shown in Figure 4B, RA (100-300 μM) had no impact on the viability of human PCIS ($n = 9$) as illustrated by stable ATP levels. In contrast to liver slices, RA had no significant influence on the expression of the investigated inflammatory and fibrotic markers in human PCIS. Out of interest, *PAI-1* level was elevated during culture with RA (Figure 5C and 5D, $n = 5$).

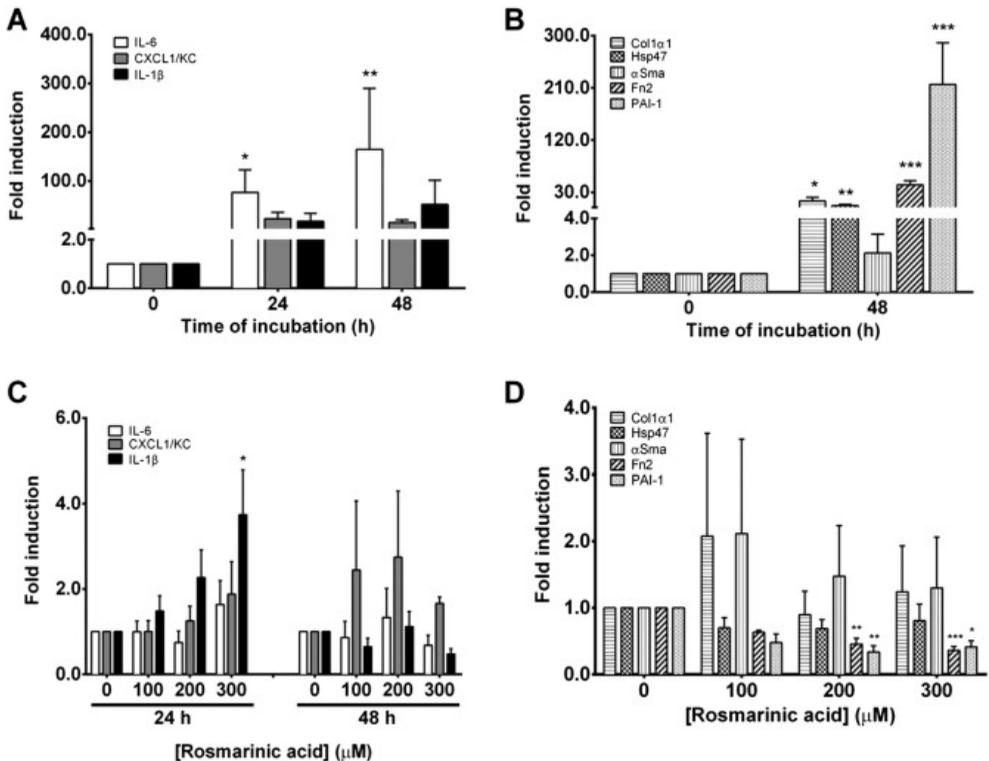


Figure 3. The effect of incubation and rosmarinic acid on murine PCLS 48 h (n = 4): (A) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1β* expressions (incubation); (B) Fibrosis markers *Col1α1*, *Hsp47*, *αSma*, *Fn2*, and *Pai-1* expressions (incubation); (C) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1β* expressions (rosmarinic acid); (D) Fibrosis markers *Col1α1*, *Hsp47*, *αSma*, *Fn2*, and *Pai-1* expressions (rosmarinic acid). Data are expressed as mean \pm SEM. ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control.

Murine PCIS

Similar to human PCIS, murine PCIS (n = 4) remain viable during culture for 48 h (Figure 2C). In addition, gene expression of *IL-6* and *CXCL1/KC*, the murine *IL-8* homologue, was up-regulated 2483 and 1721 fold respectively after 48 h incubation (Figure 6A, n = 4). Moreover, *Pai-1* levels increased more than 40 fold during culture (Figure 6B, n = 4). Of interest, when murine PCIS were treated with RA, there was a slight reduction of viability after 24 h, yet viability remained at an adequate level to study the effects of RA. However, there was a significant reduction in viability after exposure to 200-300 μ M RA for 48 h (Figure 2D, n = 4). Figure 6C (n = 4) shows that there was a significant reduction in *IL-6* expression when PCIS

were treated with 300 μM RA for 24 h. Furthermore, the expression of *IL-6* and *CXCL1/KC* also showed a significant reduction following exposure to 100 μM of RA for 48 h. In addition, we observed a concentration-dependent reduction of the studied fibrosis markers with significant effect on *Fn2* expression during 24 h, while RA had no effect after 48 h (Figure 6D, $n = 4$).

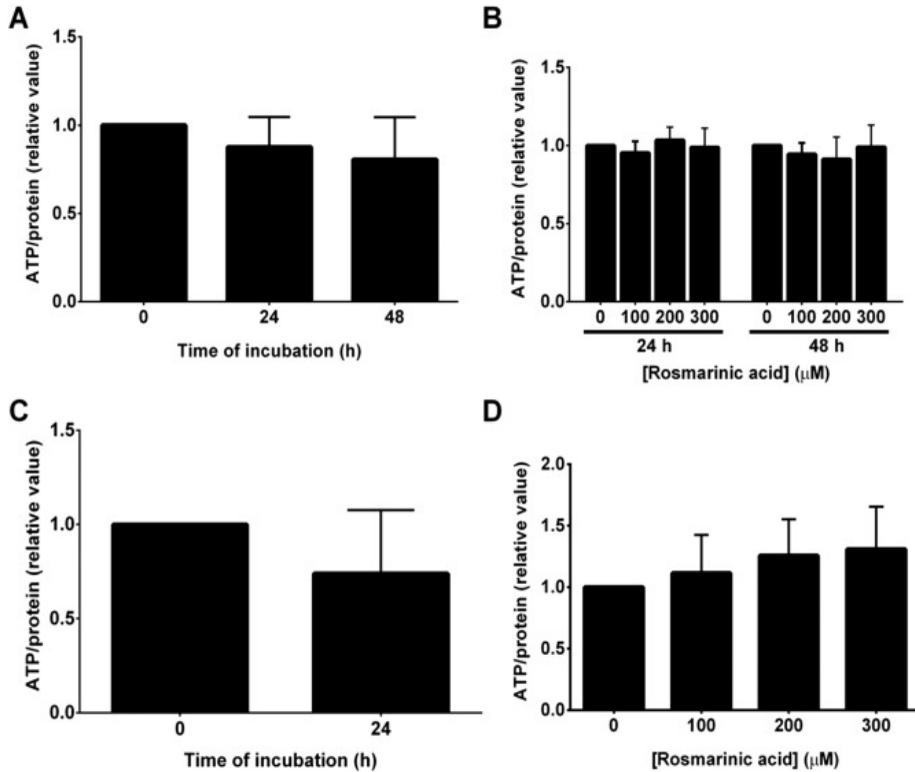


Figure 4. The effect of incubation and rosmarinic acid on human PCIS 48 h ($n = 9$) and rat PCIS 24 h ($n = 6$): (A) Human PCIS viability (relative value; incubation); (B) Human PCIS viability (relative value; rosmarinic acid); (C) Rat PCIS viability (relative value; incubation); (D) Rat PCIS viability (relative value; rosmarinic acid). Data are expressed as mean \pm SEM. Student's t-test.

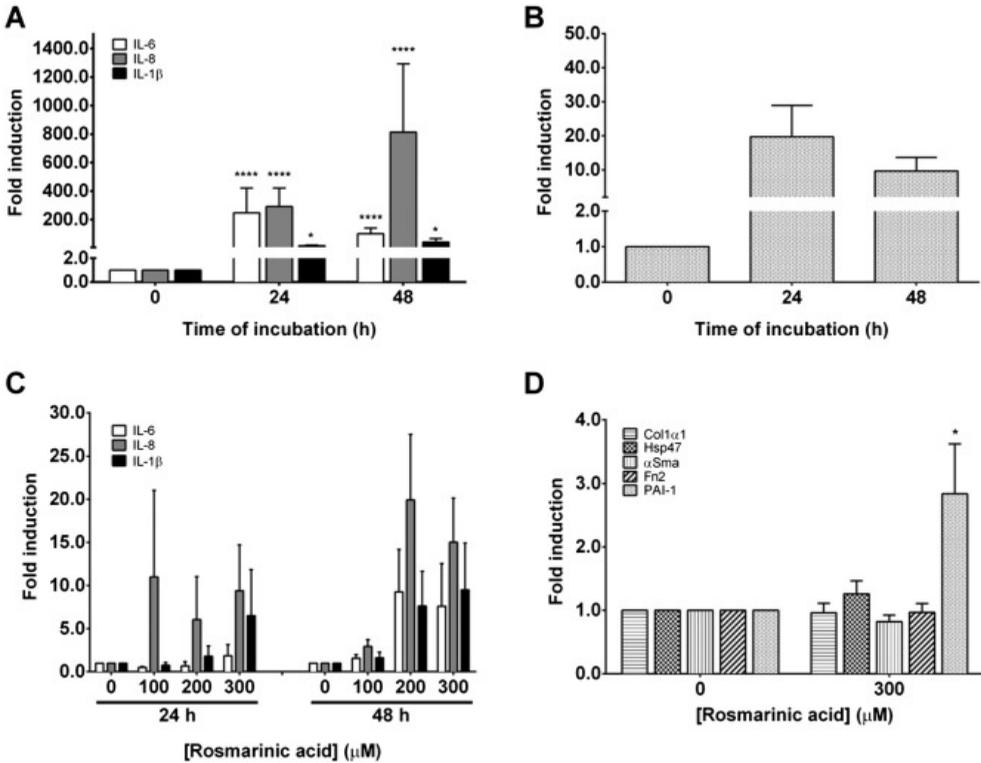


Figure 5. The effect of incubation and rosmarinic acid on human PCIS (n = 5): (A) Inflammatory markers *IL-6*, *IL-8* and *IL-1β* expressions (incubation); (B) Fibrosis marker PAI-1 expressions (incubation); (C) Inflammatory markers *IL-6*, *IL-8* and *IL-1β* expressions (rosmarinic acid); (D) Fibrosis markers *COL1α1*, *HSP47*, *αSMA*, *FN2*, and *PAI-1* expressions (rosmarinic acid). Data are expressed as mean \pm SEM. ANOVA; * $p < 0.05$, **** $p < 0.0001$ vs. control.

Rat PCIS

Rat PCIS can only be cultured for 24 h (Figure 4C, n = 4), still during this time, gene levels of *IL-6*, *IL-8* and *Pai-1* were significantly up-regulated (Figure 7A,B, n = 4). Furthermore, in contrast to the results obtained with murine PCIS, RA did not affect the viability of rat PCIS, nor did it affect the inflammatory and fibrotic response (Figure 4D, 7C, D, n = 4). Taken together, it is clear that RA elicits species-specific effects in the intestine.

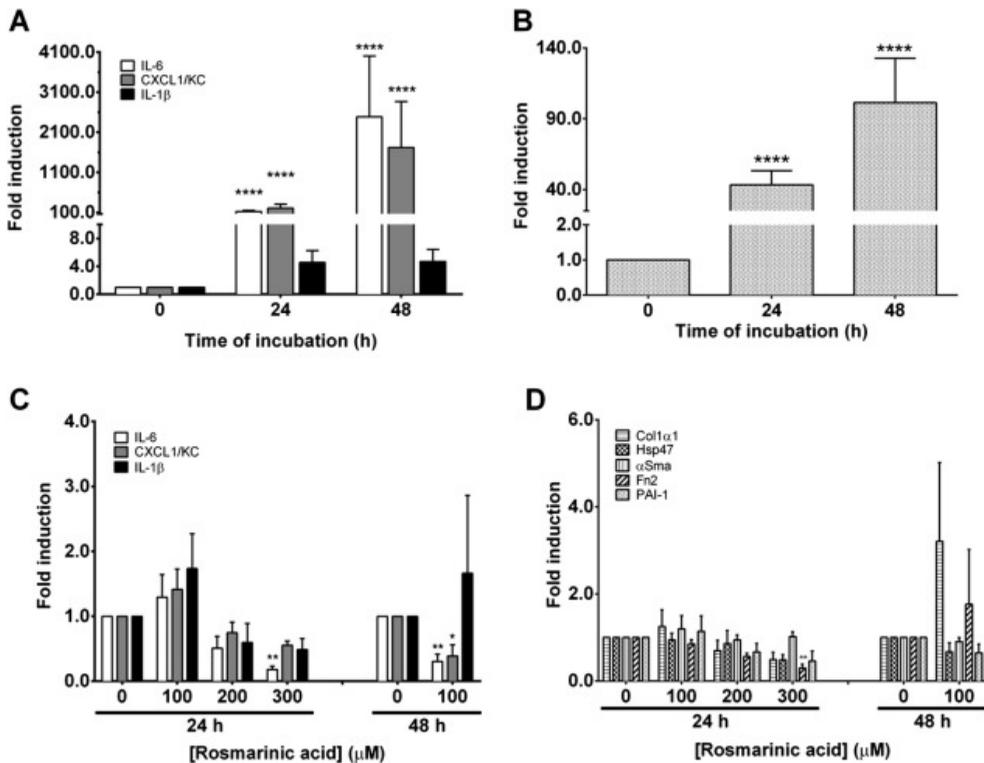


Figure 6. The effect of incubation and rosmarinic acid on murine PCIS (n = 4): (A) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1 β* expressions (incubation); (B) Fibrosis markers *Pai-1* expressions (rosmarinic acid); (C) Inflammatory markers *IL-6*, *CXCL1/KC*, and *IL-1 β* expressions (rosmarinic acid); (D) Fibrosis markers *Coll1 α 1*, *Hsp47*, *α Sma*, *Fn2*, and *Pai-1* expressions (rosmarinic acid). Data are expressed as mean \pm SEM. ANOVA; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ vs. control.

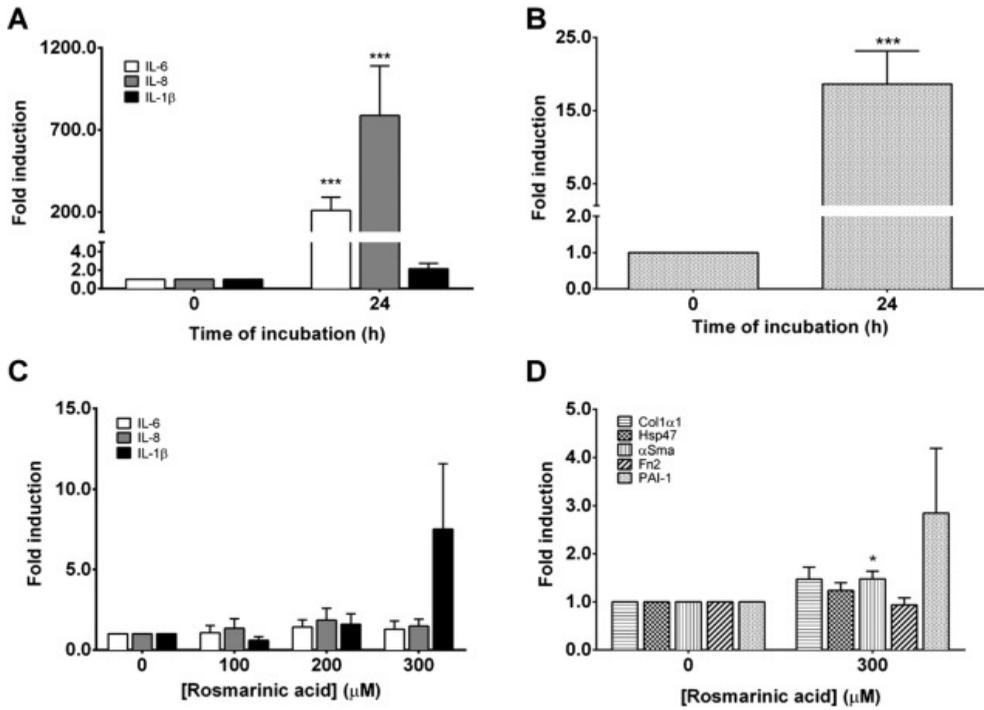


Figure 7. The effect of incubation and rosmarinic acid on rat PCIS (n = 4): (A) Inflammatory markers *IL-6*, *IL-8*, and *IL-1β* expressions (incubation); (B) Fibrosis markers *Pai-1* expression (incubation); (D) Viability (relative value) (rosmarinic acid); (E) Inflammatory markers *IL-6*, *IL-8*, and *IL-1β* expressions (rosmarinic acid); (F) Fibrosis markers *Colla1*, *Hsp47*, *αSma*, *Fn2*, and *Pai-1* expressions (incubation). Data are expressed as mean \pm SEM. ANOVA; *p < 0.05, ***p < 0.001 vs. control.

DISCUSSION

RA is an ester of caffeic acid found in a variety of plants, including the forget-me-not family. A multitude of beneficial properties have been contributed to RA, such as anti-inflammatory and antibacterial effects. Moreover, there is evidence indicating that RA might mitigate fibrosis (Domitrović et al., 2013; Hossan et al., 2014; Li et al., 2010; Scheckel et al., 2008; Westra, 2014; Westra et al., 2014a), a detrimental pathophysiological process associated with various chronic diseases. In this study, we further evaluated to biological effects of RA.

Organ toxicity of RA

Our results demonstrated that RA concentration-dependent decreased the viability of murine PCIS, whereas both rat and human PCIS were unaffected. This discrepancy might be caused by species differences in the metabolism of RA. Several studies have previously shown that there were variances in absorption, metabolism, degradation and urinary excretion of RA between rats and humans, with rats excreting more of the glucuronide conjugate and humans the sulfate conjugate (Baba et al., 2004; Baba et al., 2005; Nakazawa and Ohsawa, 1998; Nakazawa and Ohsawa, 1999). Yet, RA metabolism in mice requires further investigation. Of note, several studies have shown that RA induces cell death in a variety of human colorectal carcinoma cell lines (Cheng et al., 2011; Xavier et al., 2009) as well as cells derived from mouse adenomas (Karmokar et al., 2012). These findings suggest that, under certain circumstances, RA might indeed be toxic for intestinal cells. Conversely, our results, and previous work from our group, showed that RA is not toxic for liver slices prepared from murine, rat and human tissue (Westra, 2014; Westra et al., 2014a). Furthermore, a multitude of studies demonstrated that RA protects neural cell against apoptosis (Lee et al., 2008). Thus, RA appears to be generally non-toxic.

Anti-inflammatory effect of RA

To our knowledge this is the first study that addresses the effect of RA on the inflammation in human, rat and murine intestine. Our results with intestinal murine PCIS showed that RA only has anti-inflammatory effects in the murine intestine. As illustrated, RA potently reduced *IL-6* and *CXCL1/KC* expression. Previously, Wang

et al., showed that IL-6 is a potent proinflammatory cytokine which plays an important role in the pathogenesis of inflammatory bowel disease (IBD) (Wang et al., 2003). Our PCIS studies indicate that RA will not be a potential treatment of IBD.

Antifibrotic effect of RA

Our results demonstrated that RA can hamper fibrogenesis in murine PCLS, similar to previous observations from our lab using human and rat PCLS (Westra, 2014; Westra et al., 2014a). In contrast, RA did not affect the fibrotic response in PCIS of these species. A possible explanation for the observed discrepancy could be the mechanisms underlying fibrogenesis in both organs. In the liver, fibrosis is mainly caused by activated resident cells, whereas infiltrating immune cells are key players in the fibrotic response in the intestine (Friedman, 2008; Rieder et al., 2007). Activated hepatic stellate cells (HSCs) have numerous interactions with the immune system by means of antigen presentation, secretion of chemokines and via expression of adhesion molecules (Friedman, 2008), and they produce the majority of the ECM components associated with liver fibrosis (Wynn and Barron, 2010). On the other hand, intestinal fibrosis is mainly caused by damaging processes that elicit infiltration of immune cells, which will ultimately result in destruction of the mucosal and submucosal layers via oxidant activity (Rieder et al., 2007). Thus, RA might directly affect profibrotic resident cells (*i.e.* HSCs) in PCLS, thereby reducing the fibrotic response, whereas RA fails to target the effector cells in PCIS. Further research is needed to elucidate whether RA can mitigate intestinal fibrosis in a co-culture model using PCIS and activated macrophages.

Taken together, our results clearly demonstrate that RA has potential as a therapeutic agent for the treatment of liver fibrosis. In addition, RA appears to elicit anti-inflammatory and antifibrotic effects in murine PCIS. Conversely, these beneficial effects were not observed in human and rat PCIS. Thus, the advantageous effects of RA are clearly organ- and species-specific.

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